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Via della Ricerca Scientifica, 00133 Rome (Italy)
Tel/FAX: +39 06 72594350 FAX: +39 06 2023500
E-mail: aminoacids@bio.uniroma2.it

Amine Oxidases

Phenylpyrrolylethanoneamines related to kathinone, endowed with potent and highly selective anti-MAO-A activity

Enzo Agostinelli[1], **Paola Turini**[1], **Olivia Befani**[1], **Alessandro Martone**[1], **Roberto Di Santo**[2], **Roberta Costi**[2], **Marino Artico**[2], **Alessandra Roux**[2], **Francesco La Torre**[3], **Rossella Ferretti**[3], **Bruno Gallinella**[3], and **Roberto Cirilli**[3]

[1] Dipartimento di Scienze Biochimiche, Università di Roma "La Sapienza", P.le Aldo Moro 5, 00185 Roma, Italy
[2] "Istituto Pasteur-Fondazione Cenci Bolognetti", Dipartimento di Studi Farmaceutici, and
[3] Laboratorio di Chimica del Farmaco, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Roma, Italy

MAOs are FAD-containing enzyme located in the outer membrane of mitochondria of several cells. These enzymes have been classified into two major isoforms, MAO A and MAO B, on the basis of their amino acid sequence, substrate and inhibitor specificities. MAO A catalyzes the oxidative deamination of serotonin, adrenaline and noradrenaline and is selectively inhibited by clorgyline and moclobemide. The isoform B deaminates β-phenylethylamine and benzylamine and is inhibited by L-deprenyl. A role of Ile-335 in MAO A and Tyr-326 in MAO B, in determining substrate specificities in human enzyme, was suggested. The structure of human MAO-B was recently solved. Since the mitochondrial MAOs are involved in the metabolism of the biogenic monoamine neurotransmitters, both isoforms could be involved in psychiatric and neurological disorders. From a clinical point of view, the initial interest in administering MAO inhibitors, for the treatment of depression, faded because of their undesired effects due to an irreversible mechanism of action. Recently, the synthesis of new inhibitors led to a renewed interest to prepare and study selective and reversible molecules. Pursuing our studies on pyrrole analogs of kathinone, a natural product found in *kath* extracts, we tested against MAOs a series of 2-amino-2-phenyl-1-(1H-pyrrol-2-yl)ethanones related to moclobemide and brofaromine. The compounds were tested on bovine brain mitochondria. The activity of both isoforms, was determined by a fluorimetric method, using kinuramine as substrate and in presence of the specific inhibitors: L-deprenyl 1 μM to estimate the MAO A activity and clorgyline 1 μM to assay the MAO B. All test compounds showed good inhibitory activity and high selectivity against MAO-A. The most potent MAO-A inhibitor was the levo-pyrrolidinyl derivative which showed a Ki (M) $= 3.5 \times 10^{-9}$.

A kinetic study on cobalt(II)-substituted bovine serum amine oxidase

Andrea Bellelli and **Enzo Agostinelli**

Department of Biochemical Sciences "A. Rossi Fanelli", University of Rome "La Sapienza" and CNR, Centre of Molecular Biology, Piazzale A. Moro 5, 00185 Rome, Italy

Cu^{2+}-containing amine oxidases are mostly homodimeres. The active site also contains topa quinone (TPQ), derived from the copper-catalysed oxidation of a tyrosine residue. In BSAO phenylhydrazine reacts with one TPQ per dimer, whereas other reagents rapidly bind one TPQ, then slowly bind a second one. BSAO is active on primary amines, which are oxidized by dioxygen to the aldehyde, H_2O_2 and NH_3. The role of the metal cofactor in the catalytic mechanism of BSAO is not clear. The TPQ reoxidation by oxygen, can be achieved not only by copper but also by other metals, such as Co^{2+}. The change of the metal in the active site has an appreciable influence on the kinetic behaviour. With several substrates, Km and Kc, measured for Co^{2+}-BSAO, are different from those for native BSAO. In this study, the presteady-state and steady-state kinetics of Co^{2+}-BSAO are investigated by stopped-flow transient spectroscopy and compared with Cu^{2+}-BSAO. We have observed that the Co^{2+}-derivative reacts with the substrate with a second order rate constant lower than that of the native enzyme, and a Km values for O_2 much higher than that of the native Cu^{2+}-BSAO. The results show that the metal is involved in the mechanism of reoxidation of the reduced TPQ quinolamine; however, it is clear that reversible oxidation and reduction of the metal is not required in this reaction, since this does not occur in Co^{2+} and therefore we suggest that the metal plays a structural role. Finally, we observe that the kcat of Co-BSAO is not significantly lower than that of Cu-BSAO, thus demonstrating that the efficiency of the catalytic event itself does not depend on the nature of the metal ion.

Spectroscopic studies of the reaction between copper-containing serum amine oxidase and lipoic acid hydrazide

Enzo Agostinelli[1], **Laura Dalla Vedova**[1], **Valentina Giovannini**[1], **Enrico Morera**[2], and **Giorgio Ortar**[2]

[1] Department of Biochemical Sciences "A. Rossi Fanelli" and
[2] Department of Pharmaceutical Studies, University of Rome "La Sapienza", Piazzale Aldo Moro 5, 00185 Rome, Italy

Bovine serum amine oxidase (BSAO) (EC 1.4.3.6) catalyses the oxidative deamination of primary amines to the corresponding aldehydes, with production of ammonia and hydrogen peroxide. The stoichiometric addition of lipoic acid hydrazide to BSAO in buffer phosphate 0.1 M, pH 7.2, induced spectral changes in the visible and near u.v. region. An intense new absorption band was formed at about 348 nm. The appearance of the 348 nm band paralleled the decrease of the 480 nm absorption, corresponding to the reduction of the enzyme organic cofactor, 6-hydroxydopaquinone. Further addition of hydrazide-derivative up to 3-fold excess did not cause absorbance change. The formation of the adduct with BSAO was immediate and the time required at 25°C for complete reaction was about 3 h ($\varepsilon = 12\,000\,M^{-1} \cdot cm^{-1}$). Complete loss of enzymatic activity was observed in these conditions, after 30 min. Specific activity was not restored after 24 h dialysis at pH 7.2 in the same buffer phosphate. At pH 5.8, the optical spectrum showed an $\varepsilon = 8700\,M^{-1} \cdot cm^{-1}$ and the reaction of formation of the adduct was slower. The loss of the enzymatic activity, was partially recovered (about 35%) after 48 h of dialysis in buffer phosphate pH 5.8.

The aim of this study is to test the effect on reactivity and spectroscopic properties of lipoic acid hydrazide and some of its derivatives, respectively in view of possible pharmacological use and of an understanding of the protein molecular structure.

Structural and mechanistic studies of copper-containing amine oxidases

David M. Dooley

Department of Chemistry & Biochemistry, Montana State University, Bozeman, MT 59717, USA

New structures of complexes between substrate analogues, mechanism-based inhibitors, and small molecules with amine oxidases from

Arthrobacter globiformis and *Pichia pastoris* will be presented. The results from concurrent kinetics and spectroscopic experiments, probing the interactions of exogenous molecules with the enzymes, will also be discussed. Mechanistic inferences from the correlation of structural and kinetics data will be summarized.

Tyramine as substrate and inhibitor of lentil Copper/TPQ amine oxidase

Silvia Longu[1], **Rosaria Medda**[1], **Alessandra Padiglia**[1], **Giovanni Floris**[1], **Alessandro Finazzi Agrò**[2], and **Jens Z. Pedersen**[3]

[1] Department of Applied Sciences in Biosystems, University of Cagliari, Cagliari, Italy
[3] Departments of Biology and
[2] Experimental Medicine, University of Rome "Tor Vergata", Rome, Italy

Copper/TPQ amine oxidases (amine oxygen oxidoreductase deaminating, copper containing; E.C. 1.4.3.6) are soluble dimeric enzymes in which each subunit contains one tightly bound Cu(II) and one tyrosine-derived cofactor 6-hydroxydopa quinone as prosthetic groups. In plants amine oxidases catalyze very efficiently the oxidative deamination of diamines and polyamines with the formation of the corresponding aldehydes, ammonia and hydrogen peroxide. Several amine compounds are substrates and inhibitors of plant amine oxidases like 1,4-diamino-2-butyne, the alkylamines 2-bromo ethylamine and 2-chloro ethylamine, and indoleamines such as tryptamine, 5-hydroxytryptamine, and 5-methoxytryptamine. Tyramine is an important compound in the plant kingdom obtained by decarboxylation of tyrosine and involved in the biosynthesis of some alkaloids and in the formation of hydroxycinnamoyl amines. The latters are widely distributed metabolites and considered to be integral components of plant defence responses to pathogens. Lentil seedling amine oxidase catalyzes the oxidative deamination of tyramine, but the enzyme is irreversibly inactivated in the course of the reaction. The mechanism by which tyramine inhibits lentil amine oxidase involves the attack of the p-hydroxyphenylacetaldehyde (PHA) product on the ε-amino group of lysines to form a Schiff base adduct followed by the formation of a covalent derivative. In particular the adduct PHA-Lys296 at the active site might cause structural perturbation of the substrate channel leading to the irreversible inactivation of the enzyme.

Gene organization and molecular modeling of copper amine oxidase from *Aspergillus niger*

Ivo Frébort[1], **Marek Šebela**[1], **Shun Hirota**[2], **Mamoru Yamada**[3], **Hisanori Tamaki**[4], **Hidehiko Kumagai**[4], **Osao Adachi**[3], and **Pavel Peč**[1]

[1] Department of Biochemistry, Palacký University, Olomouc, Czech Republic
[2] Kyoto Pharmaceutical University, Japan
[3] Department of Biological Chemistry, Yamaguchi University, Japan
[4] Division of Applied Life Sciences, Kyoto University, Japan

Amine oxidase AO-I from *Aspergillus niger* AKU 3302 was reported to contain topa quinone as a cofactor, however, the study on *p*-nitrophenylhydrazine derivatized enzyme and purified active site peptides showed the presence of a carboxylate ester linkage of TPQ to a glutamate. The catalytic functionality of such a cross-linked cofactor has been shown unlikely by spectroscopic and voltammetric studies on synthesized model compounds. We have obtained resonance Raman spectra of native and substrate reduced AO-I showing that the catalytically active cofactor is unmodified TPQ. The primary structure of the enzyme (GenBank U31869) has been reviewed and updated by repeated isolation and sequencing of AO-I cDNA. This allowed rectification of several errors that account for previously reported low homology to other amine oxidases in the regions around copper binding histididyl residues. The results were confirmed by cloning the *ao-1* structural gene (GenBank AF362473). Analysis of the gene 5′-upstream region of the gene revealed potential binding sites for an analogue of NIT2, the nitrogen metabolism regulatory protein found in *Neurospora crassa* and other fungi. Molecular structure of AO-I was modeled by a comparative method using published crystal structures of amine oxidases as templates.

Inhibitor binding studies to *E. coli* amine oxidase

P. Knowles[1], **C. Kurtis**[1], **M. McPherson**[1], **J. Murray**[1], **S. Phillips**[1], **C. Saysell**[1], **W. Tambyrajah**[1], **C. Wilmot**[1], **D. Brown**[2], **D. Dooley**[2], **M. Rogers**[2], and **M. Mure**[3]

[1] Astbury Centre for Structural Molecular Biology, University of Leeds, UK
[2] Montana State University, USA
[3] University of California, Berkeley, USA

Amine oxidases, containing copper and a protein-derived quinone as cofactors, are widely distributed in *Nature* and there have been many studies of their structure and function. Phenylhydrazines have frequently been used as substrate analogues to study the chemistry involved in the catalytic cycle of these amine oxidases. However, despite extensive effort in several laboratories, the chemistry is still controversial. Our recent studies on the irreversible binding of 2-hydrazinopyridine to *E. coli* amine oxidase (ECAO) will be described.

Amphetamines have been shown to be reversible competitive inhibitors of copper/quinone amine oxidases. Our structural and kinetic studies of the binding of the amphetamine tranycypromine to wild type and mutational variants of ECAO have provided a greater insight of the chemistry involved.

Amphetamines are used clinically as antidepressants where their perceived action involves inhibition of flavin-containing amine oxidases. Their use as drugs is limited by their multiple side effects, some of which may be due to inhibition of members of copper/quinone amine oxidase.

3-(1*H*-pyrrol-2- and -3-yl)-2-oxazolidinones as novel potent and selective monoamine oxidase type A inhibitors

A. Mai[1], **I. Cerbara**[1], **S. Valente**[1], **M. Artico**[1], **G. Sbardella**[2], **O. Befani**[3], **P. Turini**[3], and **E. Agostinelli**[3]

[1] Dipartimento di Studi Farmaceutici, Università degli Studi di Roma "La Sapienza", P. le A. Moro 5, 00185 Roma, Italy
[2] Dipartimento di Scienze Farmaceutiche, Università degli Studi di Salerno, via Ponte Don Melillo, 84084 Fisciano (SA), Italy
[3] Dipartimento di Scienze Biochimiche "A. Rossi Fanelli" and Centro di Biologia Molecolare del CNR, Università degli Studi di Roma "La Sapienza", P.le A. Moro 5, 00185 Roma, Italy

Monoamine oxidase (MAO) is a FAD-containing enzyme located in the outer membrane of mitochondria. Two MAO isoforms (MAO-A and MAO-B) have been distinguished till now, catalyzing oxidative deamination of endogenous monoamines in brain and in peripheral tissues. MAO inhibitors (MAOIs) represent a useful tool for treatment of several neurological diseases. In particular, selective MAO-A inhibitors are used as antidepressant and antianxiety drugs and are claimed to protect neuronal cells against apoptosis, and selective MAO-B inhibitors can be used in the treatment of Parkinson's disease. When compared with older, non selective MAOIs, these MAOIs exhibit a more favourable tolerabil-

ity profile resulting clinically effective agents in many neuropsychiatric and affective disorders. Nevertheless, they irreversibly inhibit both MAO forms. Modern search in the anti-MAO field is now directed towards the discovery of new reversible MAOIs, selective against one isoenzyme.

We recently reported on the synthesis and biochemical evaluation of 5-substituted-3-(1*H*-pyrrol-1-yl)-2-oxazolidinones, a new class of potent and selective inhibitors of MAO-A (A. Mai et al., *J. Med. Chem.* 2002, *45*, 1180). The very interesting results obtained with such compounds prompted us to design, synthesize and test isomeric 3-(1*H*-pyrrol-2- and -3-yl)-2-oxazolidinone derivatives, with the aim to explore their potential anti-MAO activities. In such compounds the 2-oxazolidinone ring has been placed at C_2 or C_3 position of the pyrrole ring, while groups with different steric hindrance have been introduced on the pyrrole N_1 position. Enzyme inhibiting assays show that new compounds are highly active as reversible anti-MAO-A agents, being the 3-(1H-pyrrol-3-yl)-2-oxazolidinones the most MAO-A selective agents with a K_i value against MAO-A in the range of 10^{-9} M, a K_i against MAO-B in the range of 10^{-4} M, and the A-selectivity index higher than 100,000.

Structural and mutagenesis studies of *E. coli* copper amine oxidase

Christian Kurtis, Peter F. Knowles, Simon E. V. Phillips, and **Michael J. McPherson**

Astbury Centre for Structural Molecular Biology,
University of Leeds, Leeds LS2 9JT, UK

Copper-containing amine oxidases contain a copper ion and an active site tyrosine-derived amino acid cofactor, 2,4,5-trihydroxyphenylalanine quinone (TPQ) formed by post-translational processing. The amine substrate reduces the enzyme with release of product aldehyde and dioxygen then reoxidises the enzyme with release of hydrogen peroxide and ammonia. The substrate specificity of amine oxidases from different sources varies, and the enzyme from *E. coli* (ECAO) has a preference for aromatic amines such as β-phenethylamine. In the active site of ECAO there is a tyrosine residue Y381 close to the substrate entry channel. We have explored the role of this residue through site-directed mutagenesis and solution studies with different substrates. Another important question is the role of the copper in amine oxidase. Copper has been clearly demonstrated to play a redox role during the single turnover formation of the TPQ cofactor from tyrosine, but the role of the metal during turnover is less clear. We have undertaken a series of metal replacement studies with ECAO and have determined the structures of the resulting proteins allowing us to correlate spectral and activity measurements with protein structural data. Results from these studies will be presented.

Kinetic studies by amperometric electrode on bovine serum amine oxidase

K. Punakivi[1], **F. Mazzei**[2], **S. Montilla**[3], **E. Podestà**[3], **E. Agostinelli**[1], and **C. Botre**[3]

[1] Department of Biochemical Sciences "A. Rossi Fanelli",
[2] Department of Chemistry and Technology of Biologically Active Substances, and
[3] Department of Pharmacology and General Physiology,
University of Rome "La Sapienza", Piazzale Aldo Moro 5, 00185 Rome, Italy

Copper-containing amine oxidases (EC 1.4.3.6), catalyse the oxidative deamination of aliphatic and aromatic amines to the corresponding aldehydes, hydrogen peroxide and ammonia. They are mostly homodimers and contain two Cu^{II} ions and one or two molecules of the cofactor topa chinone (TPQ).

In this research we evaluate the potential use of the amperometric electrodes for studies of the enzymatic kinetics. In particular, the work shows a method for the determination of the kinetic analysis on bovine serum amine oxidase (BSAO) by means of an electrochemical sensor. The experiments were performed using BSAO free dissolved in buffer phosphate pH 7.2 as well as with BSAO immobilized on an adequate membrane and coupled with an amperometric hydrogen peroxide electrode. The characterization of the main electrochemical features of the two sensing devices have been evaluated in the presence of several BSAO substrates: spermine, spermidine and benzylamine.

The results have been elaborated by means of a specific software, in order to obtain the main kinetic parameters of the BSAO *versus* the different substrates. Then, the same experiments have been realized to determine the inhibition constant (Ki) in the interaction between BSAO with phenylhydrazine and benzylhydrazine inhibitors.

The electrochemical methods are suitable either for the evaluation of the catalytic kinetic of BSAO, or for the determination of its inhibition parameters. A comparison between the main characteristics of these methods and those previously employed for the same purposes has been done.

N-Benzyl and *N*-propargyl 1*H*-pyrrole-2-carboxyamides, as simple and effective monoamine oxidase inhibitors

Romano Silvestri[1], **Marino Artico**[1], **Giuseppe La Regina**[1], **Gabriella De Martino**[1], **Silvio Massa**[3], **Olivia Befani**[2], **Marianna Palumbo**[2], **Paola Turini**[2], and **Enzo Agostinelli**[2]

[1] Dipartimento di Studi Farmaceutici e
[2] Dipartimento di Scienze Biochimiche "A. Rossi-Fanelli"
Centro di Biologia Molecolare del CNR-, Università degli Studi di Roma "La Sapienza", P.le Aldo Moro, 5, I-00185 Roma, Italy
[3] Dipartimento Farmaco Chimico Tecnologico, Università degli Studi di Siena, Via Aldo Moro, I-53100 Siena, Italy

Monoamine oxidase (MAO) is one of the most important enzyme involved in the neurotransmitter catabolism in CNS and peripheral tissues. The two major isoforms known MAO-A and MAO-B are involved in psychiatric and neurological disorders, such as depression and the Parkinson's disease (PD), respectively. Moclobemide belongs to the reversible and selective MAO-A inhibitors, which show antidepressant effect comparable to that of TCAs, SSRIs and to the irreversible MAO inhibitor tranylcipromine in the treatment of the main depressive illness. Recent investigations suggest that MAO A inhibitors could have a role as antiapoptotic agents in neurodegenerative diseases. Furthermore studies outlined the need for new reversible and safer MAO B inhibitors in the therapy of PD. Focusing our research in this field, we synthetized new and simple MAO inhibitor molecules. The benzene ring was replaced by a pyrrole nucleous resembling some structural features of moclobemide or selegiline. Compounds were tested on bovine brain mitochondria and MAO A and B activity was determined by a fluorimetric method, performed using kinuramine as substrate. The activity of both isoforms has been selected adding to the incubation mixture their specific inhibitors: 1 μM L-deprenyl has been used to estimate the MAO A activity, while 1 μM clorgyline to detect the B isoform. The assays showed relevant inhibitory activity. 2-(*N*-Methyl,*N*-propargyl)aminomethyl-1*H*-pyrrole was the most effective although not highly selective inhibitor within the series (Ki (M) MAO-A 5.4×10^{-9} and Ki (M) MAO-B 2.0×10^{-8}, $Ki_{MAO\text{-}B}/Ki_{MAO\text{-}A}$ SI = 3.7). 1*H*-Pyrrole-2-carboxyamido derivatives showed $Ki_{MAO\text{-}B}/Ki_{MAO\text{-}A}$ Selectivity Indexes between = 1.2 and 2500. The most selective MAO-B inhibitor was 2-(*N*-Benzyl,*N*-methyl)aminomethyl-1*H*-pyrrole which showed Ki (M) MAO-A

3.5×10^{-6} and Ki (M) MAO-B 2.0×10^{-8}, $Ki_{MAO\text{-}B}/Ki_{MAO\text{-}A}$ SI = 0.057.

Activators of polyamines oxidative desamination as potential anticancer agents

T. V. Fedoronchuk, K. A. Golamazova, K. A. Miroshnicova, S. P. Syatkin, and **V. A. Frolov**

Russian People's Friendship University, Moscow, Russia

Oxidative catabolism of the higher polyamines spermidine and spermine is accomplished by the concerted action of two different enzymes. One of them is peroxysomal flavin-adenine-dependent polyamine oxidase, which oxidizes N^1-acetylspermine to spermidine and 3-acetamidopropanal, and N^1-acetylspermidine to putrescine and 3-acetamidopropanal respectively, for excreting higher polyamines out of cell.

Investigating rodent hepatomaes 22a, 27, 46, 48, 60, 61 we noticed that in cancer cells polyamine oxidase activity normally is very small or not detected. Such enzyme activity depression was primary for increasing the rate of polyamine biosynthesis. It is possible that it is the way cancer cells defend their own metabolism against toxic products of polyamine oxidase action.

With the aim of cancer therapeutic and prevention strategies we chose three new substances within new synthesized polyamine analogs, modified by uracils. These substances potentiated polyamine oxidase activity in cells of rat regenerating liver in 4–8 times and dramatically decreased polyamine levels in cell-free test systems from rodent hepatomaes and in cancer cells. However these analogs shown to have no citotoxicity on carcinoma ovary cells. Thus, these polyamine analogs activating polyamine oxidase may be of a great interest as potent new anticancer agents.

Stereospecificity of α-proton abstraction by copper amine oxidase is defined by conformation of substrate Schiff-base intermediate formed in the active site

Yen-Chen Chiu, Toshihide Okajima, Shun'ichi Kuroda, and **Katsuyuki Tanizawa**

Institute of Scientific and Industrial Research, Osaka University, Ibaraki, Osaka 567-0047, Japan

The catalytic reaction of copper amine oxidase comprises two half-reactions, divided on the basis of the redox state of its organic cofactor (topa quinone; TPQ) that is post-translationally derived from an integral amino acid residue. In the initial reductive half-reaction, substrate amine reacts with the C5 carbonyl group of the oxidized TPQ, forming the substrate Schiff base (SSB). An invariant Asp located close to TPQ abstracts an α-proton of substrate, converting SSB into the product Schiff base (PSB). Despite the highly conserved architecture of the active site including TPQ, the catalytic base (Asp), and the Cu site, stereospecificity of the α-proton abstraction differs among the enzymes from various sources. To identify the determinant of this stereospecificity, we first analyzed the process of α-proton abstraction by the enzyme from *Arthrobacter globiformis* and its variant, in which the catalytic base (Asp298) has been mutated into Ala. Although the catalytic efficiency of D298A decreased by about 10^6 folds as compared to the activity of wild type, the pro-*S* α-proton of substrate is abstracted stereospecifically both with the wild type and D298A, indicating that the presence of the catalytic base is not a direct determinant of stereospecificity. Taking advantage of the marginal activity of D298A mutant, we then characterized spectrophotometrically and crystallographically the reductive half-reaction intermediates, SSB and PSB. The structure of SSB determined for the substrate-soaked D298A crystals led to an important suggestion that the stereospecificity of α-proton abstraction is defined intrinsically by the conformation of SSB formed in the active site.

Amine oxidases and amine oxidase inhibitors

Keith F. Tipton[1], **Jeff O'Sullivan**[1], **Colin B. Seymour**[2], **Robert Mooney**[2], and **Carmel Mothersill**[2]

[1] Department of Biochemistry, Trinity College, Dublin 2, Ireland

[2] Radiation and Environmental Science Centre, Dublin Institute of Technology, Kevin St, Dublin 8, Ireland

The monoamine oxidases (EC 1.4.3.4; MAO) and the, so-called, semicarbazide-sensitive amine oxidases (EC 1.4.3.6; SSAO) differ in their cofactors, inhibitor sensitivities and tissue distributions, but catalyse the same overall reaction and overlap to some extent in substrate specificities. However, at the present state of our knowledge, it appears that SSAO has a greater diversity of alternative cellular functions. The extent to which one enzyme may substitute for the other appears rather small, although SSAO may be able to compensate to a small extent under conditions where MAO is inhibited and there is a transient increase in its activity following MAO inhibition. Unfortunately selective and specific SSAO inhibitors ore not readily available. Inhibitors of MAO-A are antidepressants, whereas MAO-B inhibitors are valuable in the treatment of Parkinson's disease and, perhaps, some other neurodegenerative conditions. Some inhibitors of MAO-B have been reported to slow the progression of Parkinson's disease by a 'neuroprotective' or 'neurorescuing' action which is independent of their MAO inhibitors action. This protective action is not restricted to neurones, since cultured peripheral cells (e.g., HaCaT) and tissue explants are protected against radiation damage (Co-60 gamma irradiation) by compounds such as (−)-deprenyl (selegiline). Protection involves, *inter alia*, elevation of the antiaptotic factor Bcl-2, This protection is not observed with tumourigenic cells (e.g., HaCaT-ras, HPV-G) or explants, since these already have high levels of Bcl-2. This effect also increases the viability of nontumourigenic cells in culture and may protect such cells against the effects of some cytotoxins used in cancer chemotherapy.

Endothelial semicarbazide-sensitive amine oxidase serves as an adhesion molecule controlling leukocyte trafficking

G. G. Yegutkin, M. Salmi, and **S. Jalkanen**

MediCity Research Laboratories, University of Turku, FIN-20520 Turku, Finland

Endothelial cells express copper-containing semicarbazide-sensitive amine oxidases (SSAO; EC 1.4.3.6) on their surface that have been attributed to the human vascular adhesion protein (VAP-1). The aim of this study was to evaluate the functional interplay between enzymatic functions of VAP-1/SSAO molecule and its ability to mediate leukocyte-endothelial adhesion. Leukocytes efficiently roll and bind to the cultured rabbit endothelial monolayer as determined by flow chamber assay, and their trafficking was modified in the presence of SSAO inhibitors. Fluorometric enzyme assay further confirmed the existence of endothelial SSAO capable of deaminating various amines with the following rank order of substrate potency: benzylamine > beta-phenylethanolamine > methylamine > tryptamine > tyramine. Importantly, low but significant semicarbazide-sensitive formation of hydrogen peroxide was also observed during lymphocyte-endothelial coincubation even in the absence of exogenous amines. We suggest that SSAO activity is a

characteristic function of the VAP-1 molecule in vascular endothelium and that the involvement of VAP-1/SSAO in the extravasation process can be defined by local oxidation of currently undefined lymphocyte cell-surface associated substrates. This hypothesis was further confirmed by using synthetic lysine-containing peptide fitting into the groove and capable of concurrently inhibiting both SSAO activity and leukocyte-endothelial adhesion.

The involvement of semicarbazide-sensitive amine oxidase in vascular disorders and obesity in spontaneous obese and diabetic KKAy mice

Peter H. Yu, Y. L. Deng, M. Wang, E. H. Fan, and **Gubisne-Haberle**

Neuropsychiatry Research Unit, Department of Psychiatry, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

Semicarbazide-sensitive amine oxidase (SSAO) is located in vascular systems and adipocyte and catalyzes the deamination of methylamine and aminoacetone producing toxic formaldehyde and methylglyoxal, respectively. SSAO activities increased in patients with diabetic mellitus and in patients with atherosclerosis, heart failure and cerebral infarct. The deaminated products, such as the toxic aldehydes, have been proposed to initiate protein cross-linkage and aggregation, exacerbate advanced glycation end products, and cause endothelial injury. The KKAy mouse, a strain possessing features closely resembling those of non-insulin-dependent diabetes mellitus, has been employed to substantiate this hypothesis. Vascular lesions were induced *via* chronic feeding of a high cholesterol diet. SSAO inhibitors effectively inhibited aorta SSAO activity, and caused a substantial increase in urinary methylamine, as well as a decrease in formaldehyde and methylgloxal levels. Inhibition of SSAO also reduced oxidative stress, as shown by reduction of malondialdehyde excretion, albuminuria, proteinuria, the number of atherosclerotic lesions, and weight gain in animals following the chronic treatment with a high cholesterol diet. Increased SSAO-mediated deamination could be involved in the cascade of atherogenesis related to diabetic complications. We also found that selective a SSAO inhibitor induces mild hyperglycemia and reduce obesity. This seems to be related to adipose SSAO, since hydrogen peroxide generated from SSAO-mediated deamination regulates adipose glucose transport and seemingly related to adipocyte differentiation. The relationship of SSAO to the vascular disorders is consistent with its role as an adhesion molecule (VAP-1) in the process of inflammation.

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Protein cross-linkage induced by formaldehyde derived from oxidative deamination of methylamine catalyzed by semicarbazide-sensitive amine oxidase (SSAO)

Diana Gubisne-Haberle and **Peter H. Yu**

Neuropsychiatry Research Unit, Department of Psychiatry, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

SSAO is a copper-containing enzyme, which is distributed in and on the surface of vascular smooth muscles, endothelial cells and adipocytes. Methylamine and aminoacetone are known endogenous substrates for SSAO. The deamination of these amines leads to production of toxic formaldehyde and methylglyoxal, respectively as well as hydrogen peroxide and ammonia. In humans, plasma SSAO activity is elevated in both type I and type II diabetes mellitus and in chronic heart failure. SSAO activity is also increased in different tissues of experimental diabetic animal models. Selective SSAO inhibitors can reduce atherogenesis in diabetic KKAy mice and atherosclerotic rabbits induced by high cholesterol diet. An increase in SSAO-mediated production of aldehydes has therefore been proposed causing direct or indirect damage of the vasculatures through formation of intramolecular and intermolecular protein cross-linkages, subsequent protein aggregation and plaque formation. We observed that intravenous injection of ^{14}C-methylamine would induce a long-lasting deposition of radioactivity associated to proteins in different mouse tissues; such formaldehyde-induced protein cross-linkage can be blocked by selective SSAO-inhibitors. In the present study we employ different *in vitro* and *in vivo* models and identify the SSAO-mediated formaldehyde-protein cross-linkage. Lysine residue is the primary target of formaldehyde-induced cross-linkage. Methyl-lysine, assessed by HPLC-MS, was detected in the SSAO induced formaldehyde-protein adducts. The observations support the idea, that an increase in SSAO-mediated deamination may induce protein deposition adjacent to the vascular site, where SSAO is located, which is related to vascular disorders.

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Towards the crystal structure of amine oxidase from bovine serum

Marianna Biadene[1], **Vito Calderone**[1], **Maria Luisa Di Paolo**[2], **Michela Trabucco**[1], **Roberto Battistutta**[1,3], **Adelio Rigo**[2], and **Giuseppe Zanotti**[1,3]

[1] Department of Organic Chemistry, University of Padova, Via Marzolo 1, 35131 Padova, Italy

[2] Department of Biological Chemistry of the University and ICTB, Section of Padova, Via G. Colombo 3, 35121 Padova, Italy

[3] Venetian Institute of Molecular Medicine, Via Orus 2, 35100 Padova, Italy

The structural characterization of copper-containing amine oxidases [amine-oxygen oxidoreductase (deaminating) (copper/TPQ-containing); EC 1.4.3.6] is of broad importance to understand the structure/function relationships of these enzymes that play a variety of fundamental functions in the metabolism of cellular and extracellular amines. Copper-containing amine oxidases are a class of ubiquitous enzymes that catalyze the two-electron oxidation of primary amines to the corresponding aldehydes, with reduction of molecular oxygen to hydrogen peroxide. Till now, the three-dimensional structure of the enzyme from higher animals and, in particular, from mammalian organisms has not been determined. One of the most studied among these enzymes family is bovine amine oxidase (BSAO), whose carbohydrate content account for about 4.3% of its weight.

The copper-containing amine oxidase extracted from bovine serum (BSAO) and purified to homogeneity, has been deglycosylated and crystallized. The crystals obtained belong to space group $P2_12_12_1$, with unit-cell parameters $\mathbf{a} = 77.68$ Å, $\mathbf{b} = 131.19$ Å, $\mathbf{c} = 134.00$ Å and diffract to at least 2.4 Å resolution. Native diffraction data were collected at the diffraction beamline of ELETTRA synchrotron (Trieste, Italy), at 100 K, using one crystal.

At present, the three-dimensional structure of four different amine oxidases (from *E. coli*, pea seedling, *A. globiformis* and *H. polymorpha*) is known. A relatively high homology in the amino acid sequence can be detected only in the central part of the protein, from amino acid around 290 till 700. A partial model of the dimer comprising only those amino acids was built with the ExPASy modelling server. Using that partial model, a preliminary molecular-replacement solution has been obtained with the software AMoRe. The refinement and the model building of the remaining part of the model is in progress in our laboratory.

Amino Acid and Protein Oxidation

An overview of protein oxidation

Earl R. Stadtman

Laboratory of Biochemistry, National Heart, Lung,
and Blood Institute, National Institutes of Health, Bethesda,
Maryland 20892-8012, USA

Proteins are particularly sensitive to oxidative modification by diverse reactive oxygen species (ROS). Direct oxidation of proteins by ROS can lead to fragmentation of the polypeptide chain, generation of protein-protein cross-linked derivatives, or to modification of any one of a number of amino acid residues. In addition, oxidative damage to proteins can occur indirectly by interaction of functional groups with oxidation products of polyunsaturated fatty acids and/or carbohydrates (glycation and glycoxidation) to produce biologically inactive derivatives. Highly sensitive chemical and immunochemical methods have been developed for the identification and quantitative estimation of the various kinds of oxidative protein modification. Using these techniques it has been demonstrated that the accumulation of oxidized proteins is associated with animal aging and with the development of many diseases, including atherosclerosis, cancer, cataractogenesis, Alzheimer's disease, Parkinson's disease, diabetes, uremia, amyotrophic lateral sclerosis (ALS), etc. In addition, the oxidation of proteins is implicated in protein turnover and in the regulation of some enzyme activities. Moreover, the consumption of ROS by cyclic oxidation and repair of cysteine and methionine residues of proteins may serve important antioxidant functions.

Proteomic identification of oxidatively- and nitrosatively-modified proteins in Alzheimer's disease brain: insights into potential mechanisms for neurodegeneration

D. Allan Butterfield

Department of Chemistry, Center of Membrane Sciences,
and Sanders-Brown Center on Aging, University of Kentucky,
Lexington, KY 40506, USA

Extensive oxidative stress is present in Alzheimer's disease (AD) brain, manifested as protein oxidation, lipid peroxidation, and DNA/RNA oxidation, among other indices. Amyloid β-peptide, especially the 42-mer Aβ(1-42), is thought to be central to the pathogenesis of AD, and Aβ(1-42), in ways that are inhibited by antioxidants, is able to induce neuronal protein oxidation, lipid peroxidation, and free radical formation. Protein oxidation, indexed by increased protein carbonyls, is increased in AD brain in those regions rich in Aβ(1-42), but absent in cerebellum, which is poor in Aβ(1-42). Moreover, the activity of two important enzymes, creatine kinase (CK) and glutamine synthetase (GS), were found to be dramatically decreased in these same brain regions where Aβ(1-42) is found. Although protein oxidation is present in AD brain, the identity of specifically oxidatively modified proteins remained unknown until now. Protomics allows identification of oxidatively modified proteins. Analysis of inferior parietal lobule of AD and control brain, obtained at extremely short PMI ($<$3 hours), indicated the following proteins as being specifically oxidatively modified: (a) CK; (b) GS; (c) ubiquitin carboxy-terminal hydrolase L-1 (UCH L-1); (d) α-enolase; and (e) dihydropyrimidinase related protein-2 (DRP2), also called collapsin response mediator protein 2 (CRMP-2) Specific proteins in AD brain with increased 3-nitrotyrosine (3-NT), which is formed from peroxynitrite attack on proteins, include: (a) α- and γ-enolase; (b) triosephosphate isomerase (TPI); and (c) neuropolypeptide h3 (also known as phosphatidylethanolamine binding protein, PEBP). Each of these oxidatively modified proteins can be invoked in mechanisms for neurodegeneration and/or pathology observed in AD brain, including: (a) mechanisms related to the known decreased energy utilization in AD brain (CK; α- and γ-enolase; TPI); (b) mechanims related to excitotoxicity (GS), especially when coupled to the oxidative modification of the glutamate transporter GLT-1 (EAAT2) in AD brain and by Aβ(1-42) recently reported from our laboratory; (c) mechanisms related to impaired proteasomal degradation of aggregated, misfolded, or oxidized proteins (UCH L-1); (d) mechanisms related to activation of cholineacetyltransferase (PEBP), whose activity is drastically decreased in AD brain and a protein on which Aβ(1-42)-induced lipid peroxidation leads to oxidative modification, or to increased apoptosis; and (e) mechanisms related to the known decreased dendritic length in AD neurons, with likely consequent decreased interneuronal communication (CRMP-2), which conceivably could be involved in the memory loss observed in AD patients.

This plenary lecture will present in detail these results, which exemplify an important application of the emerging techniques of proteomics to the major dementing disorder. Our proteomics findings couple the oxidative stress in AD brain to potential mechanisms of neurodegeneration.

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Oxidation and glycation protein products as inflammatory mediators in the uremic syndrome

Francesco Galli

Department of Internal Medicine, Section of Applied
and Clinical Biochemistry, University of Perugia, V. del Giochetto,
06126-Perugia, Italy

End-stage renal disease (ESRD) and particularly chronic hemodialysis patients (HD) show a typical inflammatory syndrome (IS) with the nature of a subclinical chronic disorder of the cytokine system and acute phase proteins, that strongly influences morbidity and mortality in these patients.

Uremic solutes, incidental/recurrent infections, bio-incompatibility of extracorporeal circulation and bacterial contamination of dialysis fluids are between the most important causes for IS paralleled by immune dysfunction in ESRD. However, HD subjects show increased inflammatory markers also in absence of clinical signs of infection or inflammation and these markers persist even after dialysis carried out with the most effective and biocompatible techniques. This suggests that uremic toxicity is a major contributor to IS, and that a relevant fraction of proinflammatory mediators shows a molecular weight (MW) above the cut-off imposed by standard dialysis methods (usually $<$40 KDa).

A range of ''proteinaceous'' high molecular weight solutes (HMW) accumulates in the uremic blood. Glycation and oxidation byproducts of the human serum albumin (HSA), identified as most important inflammatory mediators activate blood and tissue phagocytes engaging specific inducible scavenger receptors (SR), thus leading to increased production of reactive oxygen species and inflammatory cytokines such as IL-1-b, TNF-a and IL-6.

Paradoxically, the HMW may generate a pro-inflammatory loop in which through inflammatory cell activation they sustain their own accumulation by increased regeneration and defective turnover.

Previous work by our group has confirmed the close correlation existing between the extent of AGE accumulation and IS severity. We also characterized the blood levels of AGEs and other HMW deriving from protein oxidation in groups of patients with chronic renal failure not yet on dialysis, ESRD patients treated with different membranes and dialysis techniques, and in transplanted patients. In a recent clinical trial on high biocompatibility ''protein-leaking'' dialysers (nominal cut-off $\geq$70 KDa) we further demonstrated the pro-inflammatory role of some HMW and the positive clinical outcome of removing these toxins from the uremic blood. This

opened a new therapeutic prospect for the correction of IS in ESRD. Other strategies that could contribute to prevent HMW accumulation could be based on specific anti-oxidant and anti-inflammatory therapies.

Involvement of intracellular glutathione in zinc deficiency-induced activation of hepatic stellate cells

Akiko Kojima-Yuasa, Tomoko Ohkita, and **Isao Matsui-Yuasa**

Department of Food and Human Health Sciences, Graduate School of Human Life Science, Osaka City University, 3-3-138 Sugimoto, Sumiyoshi-ku, Osaka 558-8585, Japan

Background: Hepatic stellate cells (HSC) have the most important role in the regulation of liver extracellular matrix homeostasis and play a key role in the development of liver fibrosis. We have already reported that enhanced type I collagen expression occurs in HSC with zinc deficiency and that the expression was inhibited with zinc supplementation. However, the mechanism of zinc deficiency-induced activation of HSC is not clear. Glutathione (GSH), the major non-protein thiol in mammalian cells, is involved in many cellular functions. In this study, we investigated the effect of zinc deficiency on intracellular glutathione level in HSC.

Methods: Isolated HSC were incubated with or without zinc chelator, diethylenetriamine penta-acetic acid (DTPA). Type I collagen expression in HSC was detected by immunohistochemistry. Intracellular GSH was measured by HPLC. Cell cycle was analyzed by laser scanning cytometry.

Results: Zinc deficiency significantly reduced cell proliferation and caused an accumulation of cells in the Sub-G1 phase. Zinc deficiency caused a reduction in intracellular GSH at 8 hours after the addition of DTPA, compared with control levels. When *N*-acetyl-L-cysteine or glutathione ethyl ester was added to the culture at 6 hours after the addition of DTPA, the intensity of the staining for type I collagen in HSC was decreased to the control level.

Conclusions: The results of this study show that in HSC the chelation of zinc triggers a progression of collagen synthesis and this may be through the decrement of intracellular GSH content from 6 to 8 hours after the addition of DTPA.

Cellular thiol status-dependent inhibition of tumor cell growth by green tea extract

Isao Matsui-Yuasa, D. O. Kennedy, and **Akiko Kojima-Yuasa**

Department of Food and Human Health Sciences, Graduate School of Human Life Science, Osaka City University, 3-3-138 Sugimoto, Sumiyoshi-ku, Osaka 558-8585, Japan

Background: Green tea has shown to be active chemopreventive agents against cancer both *in vivo* and *in vitro* studies. However, the complete mechanisms underlying the inhibitory effects are not yet fully understood. In most of our previous studies, the green tea extract (GTE) and its galloyl polyphenols, epigallocatechin (EGC), have shown tumor growth inhibitory effects in Ehrlich ascites tumor cells and several mechanism have been elucidated. Notable among these include decrease in ornithine decarboxylase activity and in cellular GSH and protein SH levels, cellular thiol-dependent induction of mitogen activated protein kinases including JNK-SAPK, p38 and ERK1/2 and changes in mitochondrial integrity involving cytochrome c release, and subsequent activation of caspasae-3-like protease. In this study, we investigated the involvement of retinoblastoma protein (Rb) phosphorylation in the growth inhibitory effect of EGC.

Methods: Ehrlich ascites tumor cells were cultured in Eagle's MEM medium containing 10% FCS. The changes of cell cycle were analyzed by laser scanning cytometer. Phosphorylation of Rb was examined by western blotting.

Results: We observed that cells incubated with EGC resulted in an accumulation of a discrete sub-population of signals under the sub-G1 and G1 cell cycle regions and a decrease in S cell cycle region. Furthermore, EGC decreased hyperphosphorylated Rb levels in a dose-dependent and time-dependent manner. The effect of Rb phosphorylation decreased by EGC was abolished with N-acetylcysteine.

Conclusions: The cellular thiol-dependent mechanism evoked by EGC may provoke a useful insight into additional mechanisms governing cell cycle progression during growth arrest by green tea polyphenols.

Regulation of mercaptopyruvate sulfurtransferase activity by oxidative stress

Noriyuki Nagahara

Department of Environmental Medicine, Nippon Medical School, Tokyo, Japan

In a mercaptopyruvate pathway; one of cysteine degradation pathways, cysteine is catalyzed by cysteine aminotransferase [EC.2.6.1.3] to form mercaptopyruvate which is then, metabolized to pyruvate by mercaptopyruvate sulfurtransferase (MST) [EC.2.8.1.2]. In rat MST, Cys^{247} is a catalytic site which forms a persulfide in transsulfuration.

MST was inhibited by hydrogen peroxide or tetrathionate at about 1.2-fold concentration of enzyme and the activity could be restored by dithiothereitol (DTT). On the other hand, MST was inactivated by excess of hydrogen peroxide and DTT could not restore the activity. The results suggested that a catalytic site, cysteine was oxidized to form cysteine-sulfenic acid by low concentration of hydrogen peroxide and to form sulfenyl thiosulfate by tetrathionate. On the other hand, high concentration of hydrogen peroxide may oxidize Cys^{247} to form cysteine-sulfinic acid and -sulfonic acid which could not be reduced by DTT.

It was reported that oxidative stress increased cystathionine b-synthase activity and suppressed methionine synthase activity, resulting in facilitation of a metabolic flow from methionine to cysteine and consequently increase in thiol containing compounds, which is consistent with the results of the present study. It is strongly suggests that amphibolic metabolism of cysteine is a critical defense against oxidative stress in the cells.

LDL, misfolding and structural implications of estrogens

Tiziana Parasassi

Istituto di Neurobiologia e Medicina Molecolare – CNR, Sezione di Medicina Molecolare, Viale Marx 15-43, 00137 Roma, Italy

The secondary structure and conformation of apoB-100 on low density lipoproteins (LDL) are severely altered during oxidative modification, as monitored by the progressive loss of the α-helix structure and by the increased hydration of the apoprotein environment. Among different proposed mechanisms to account for the protection exerted by estrogens against the early onset of atheromatous lesions, the antioxidant effect attracted considerable attention. 17-β-estradiol (E2) is able to affect apoB-100 structure and conformation with a fractional increase of the α-helix structure and the induction of an overall more compact conformation. Although the free radical scavenging capacity of E2 is debated, it markedly delays the structural and conformational changes due to LDL oxidative modification. Indeed, in the presence of E2 the α-helix loss of apoB-100 is delayed and the newly acquired conformation appears more stable. We can argue that by affecting apoB-100 structure and conformation, E2 modifies the interaction of this protein with the outer lipid layer, therefore accounting for the observed delay of lipid oxidation. We can appreciate the relevance of the induction of a stable apoB-100 conformation by referring to the electronegative subpopulation of LDL, LDL(−). This subclass is considered to posses several atherogenic properties and represents the *in vivo* counterpart of *in vitro* modified LDL. LDL(−)

displays a severely altered conformation and quite a poor content in α-helix structure. In addition, LDL(−) shares its tendency to aggregation, resistance to proteolysis and cytotoxicity with amyloidogenic proteins.

Cu(II)-catalyzed cleavage and oxidation of the N-terminal region of the salivary peptide histatin-5

R. Petruzzelli[1], **M. Patamia**[2], **R. Inzitari**[3], **I. Messana**[4], **B. Giardina**[2,3], and **M. Castagnola**[2,3]

[1] Department of Biomedical Sciences, University "G. D'Annunzio" Chieti, Italy
[2] Institute for the Chemistry of Molecular Recognition, C.N.R., and
[3] Institute of Biochemistry and Clinical Biochemistry, Catholic University of Rome, Italy
[4] Department of Sciences Applied to Biosystems, University of Cagliari, Italy

DSHAKRAHGY ($H5_{1-10}$), a peptide modeling the N-terminal amino acid sequence of human salivary histatin-5, was studied in its interaction with Cu(II), at pH 7,4 and in the presence of ascorbate by electrospray ion trap mass spectrometry. The metallopeptide nature of this fragment has been previously characterized following the appearance of absorbance maximum at 525 nm in the presence of Cu(II). These spectroscopic properties were attributed to the first three amino acids DSH, corresponding to an ATCUN motif. The present experiments confirm the formation of the complex $His5_{1-10}$-Cu(II), as evidenced by the m/z 601.8 $[M\text{-}Cu]^{2+}$ ion. Moreover, after 6 min incubation with 700 μmol/L ascorbate, two new m/z 802.5 $[H5_{3-10}+H^+]^{1+}$ and 401.7 $[H5_{3-10}+2H^+]^{2+}$ fragments, corresponding to the last seven amino acids, were detected. After 1 h of incubation the presence of the oxidation products at the level of heptapeptide and the complex DSH-Cu(II) were revealed. These results demonstrate that the association copper-peptide in the presence of ascorbate is able to auto-catalyze the cleavage at the level of the ATCUN motif, followed by the oxidation of the released fragments. This activity can be related to the generation of reactive oxygen species (ROS). This behaviour is interesting because it has been recently proposed that, in the yeast, histatin-5 target is the energized mitochondrion, being its toxic effect expressed through the interaction with the respiratory chain and the production of ROS.

Specific hemin catalyzed low density lipoprotein oxidation reactions: implications for metabolic and inflammatory diseases

Jens Pietzsch

Institute of Bioinorganic and Radiopharmaceutical Chemistry, Research Center Rossendorf, Dresden, Germany

Oxidation of LDL protein and lipids by transition metal catalyzed processes is a key factor in atherogenesis. Clinical and experimental evidence suggests that these processes involve binding of hemin, a product of hemoglobin degradation, to discrete binding sites of LDL thus forming centers for redox cycling and repeated radical production. The mechanisms for these observations remain unclear. In the present study, we found that hemin binds rapidly to low density lipoprotein subfractions (LDL_1, 1.019–1.044 kg/L; LDL_2, 1.044–1.063 kg/L) with binding rates in the nM range. Spectrophotometric and fluorescence experiments indicated that the amphiphilic hemin molecule is buried in the lipoprotein surface monolayer with the carboxylic groups in contact with positively charged surface regions. *In vitro*, in the presence of H_2O_2, hemin oxidizes both LDL_1 and LDL_2 with formation of specific products of oxidation of positively charged protein amino acid residues (γ-glutamyl semialdehyde and α-aminoadipic semialdehyde) and phospholipids (1-palmitoyl-2-oxovaleroyl-*sn*-glycero-3-phosphorylcholine). The formation of these products was more than twofold higher ($p < 0.01$) when compared with oxidation systems containing free iron or copper. Hemin catalyzed LDL oxidation was inhibited by the iron-chelating agents 1,2-Dimethyl-3-hydroxypyrid-4-one and *N*,*N*-bis(2-hydroxybenzyl)ethylenediamine-N,N-diacetic acid, respectively. *In vivo*, a significantly increased formation of all three specific oxidation products could be confirmed in circulating plasma LDL_1 and LDL_2 particles obtained from patients with impaired glucose tolerance ($p < 0.05$), Type 2 diabetes mellitus ($p < 0.05$), and rheumatoid arthritis ($p < 0.01$) when compared with healthy controls. The results provide further evidence on pathophysiological relevance of hemin catalyzed LDL oxidation in metabolic and inflammatory diseases.

Assessment of metabolism of native and oxidized low density lipoprotein *in vivo*: insights from animal positron emission tomography (PET) studies

Jens Pietzsch, Ralf Bergmann, Frank Wuest, Michaela Grote, Christina Hultsch, Beate Pawelke, and **Joerg van den Hoff**

Institute of Bioinorganic and Radiopharmaceutical Chemistry, Research Center Rossendorf, Dresden, Germany

Oxidative modification of low density lipoprotein (LDL) is regarded as a crucial event in atherogenesis. Data concerning the role of circulating oxidized LDL (oxLDL) in the development of atherosclerosis are scarce. One reason for this is the shortage of methods for direct assessment of metabolism of oxLDL *in vivo*. We report an improved methodology for labelling of both native LDL (nLDL) and oxLDL with fluorine-18 (^{18}F) by N-succinimidyl 4-[^{18}F]fluorobenzoate ([^{18}F]SFB) and the use of LDL-[^{18}]FB-conjugates in dynamic PET studies in Wistar rats. For labelling experiments, pools of chemically well characterized human nLDL and oxLDL, respectively, were used. Preparation of [^{18}F]SFB was achieved within 40 min with radiochemical yields of $50 \pm 5\%$ and purity of $>95\%$ using *O*-(*N*-succinimidyl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TSTU) as activating reagent. LDL labelling with [^{18}F]SFB resulted in radiochemical yields of $30 \pm 10\%$. The method was evaluated with respect to uptake of FB-conjugated nLDL in HepG2 cells and of FB-conjugated oxLDL in primary human macrophages, respectively. Biodistribution studies revealed high *in vivo* stability for the LDL-[^{18}F]FB conjugates. The metabolic fate of LDL-[^{18}F]FB conjugates *in vivo* was delineated by PET using a dedicated small animal tomograph (microPET; spatial resolution of 2 mm). In conclusion, [^{18}F]SFB-labelling of LDL and the use of PET provide a valuable tool for assessment of metabolism of nLDL and oxLDL *in vivo*.

Activation mechanism of porphobilinogen synthase by a reducing agent

Nori Sawada, Noriyuki Nagahara, and **Tomoyuki Kawada**

Department of Environmental Medicine, Nippon Medical School, Sendagi Bunkyo-ku Tokyo, Japan

Porphobilinogen synthase (PBGS) [EC 4.2.1.24] is a metalloenzyme, which contains two zinc ions, and catalyzes condensation and dehydration of two molecules of δ-aminolevulinic acid to produce a porphobilinogen in a step of biosynthesis of a heme. It is well-known that, when an activity for PBGS is measured, the activity is increased by 2-mercaptoethanol (2-ME) probably due to reduction of oxidized cysteine residues. To elucidate the cysteine residue which was critical for activation by a reductant, we

constructed four mutant enzymes (C122A, C124A, C132A, C223A); Cys^{122}, Cys^{124} and Cys^{132} placed near a catalytic site, Lys^{252}, coordinate a zinc ion, on the other hand, Cys^{223} at an entrance of a catalytic cavity coordinates the other zinc ion.

Reduction by 2-ME increased the specific activities of wild type and C223A mutant enzymes to about 6- and 4-fold control values without treatment by 2-ME, respectively. This finding suggests that Cys^{223} is not responsible for the activation *via* reduction. On the other hand, reducing agent did not affect the activity of C132A mutant enzyme, suggesting that Cys^{132} was a critical residue for increase in the activity by reduction. The specific activities of C122A and C124A mutant enzymes were very low (9.7×10^{-4} unit/mg and 7.5×10^{-4} unit/mg, respectively). This result suggests that Cys^{122} and Cys^{124} are essential residues for catalysis of this enzyme. We are studying the oxidation state of Cys^{122}, Cys^{124} and Cys^{132} in oxidized PBGS.

Different roles of cystamine

E. Amodeo and **S. P. Solinas**

Dipartimento di Medicina e Oncologia Sperimentale,
Università di Torino, Torino, Italy

Cystamine is an aliphatic diamine containing a disulfur bridge, formed by oxidation of two sulphydryl groups of cysteamine residues, formed in the metabolism of coenzyme A. It is metabolized to several compounds, such as thiocysteamine, hypotaurine and taurine. The last one plays an important role in the synthesis of bile salts, in membrane stabilization and in neurotransmission. Moreover several and different biological roles have been assigned to cystamine: among these the most usual are a radioprotective action on DNA, an antiviral effect in cell lines infected by HIV-1 virus and an ability to modify the Ca^{++} intracellular homeostasis. In addition to these well-known effects recent results show that cystamine is involved in very interesting biological processes, distinct from the above-mentioned. In fact this diamine:

- has therapeutic effects on the neurodegenerative disorder associated with CAG repeat expansion, leading to expression of polyglutamine;
- inhibits caspase-3 activity, contributing to the protective effects described in mouse models of the Huntington disease;
- participates in the control of tissue transglutaminase activity;
- binds DNA and induces chromatin condensation.

Moreover our recent studies demonstrate that rat hepatoma cells treated with 1.0 mM cystamine lead to apoptotic death. This observation could be connected with the action of diamine oxidase on cystamine, and therefore with the production of H_2O_2, ROS and metabolites toxic for the cells.

Amino Acid Synthesis and Medicinal Chemistry

Design, synthesis and evaluation of *syn-O*-(carboran-1-yl)methyl-3-hydroxytyrosine (CMHT) and its derivatives for boron neutron capture therapy

T. Asano[1,2], **H. Kondoh**[3], and **M. Kirihata**[1]

[1] Graduate School of Agriculture and Life Sciences, Osaka Prefecture University, Sakai, Osaka 599-8531
[2] Research & Development Department, Stella Chemifa Corporation
[3] Bio-Research, Inc.

Boron neutron capture therapy (BNCT) is radiotherapy for cancer based on the nuclear capture reaction between stable boron-10 isotope (^{10}B) atom and thermal neutron. Low energy thermal neutrons hit boron-10 atoms within some boron compound which is incorporated into cancer cells, trigger the emission of alpha-particles and ^{7}Li atoms by the ^{10}B(n, alpha) ^{7}Li reaction. These particles can eradicate cancer cells selectively without serious injury to the surrounding normal tissues, as their traveling range is approximately 10 micro-meter.

The most critical point for success of BNCT is to deliver sufficient amount of ^{10}B into cancer cells than normal cells. Based on our series of synthetic studies on the analogues of boronophenylalanine (BPA) currently using BNCT, we attempted to link boronclusters, especially icosahedral-carboranes with BPA-backbone to achieve this subject.

Following this design strategy, a new boron compound, *syn-O*-(carboran-1-yl)methyl-3-hydroxytyrosine (CMHT) was designed. Our approach for the synthesis of this compound is based on a stereoselective aldol-type condensation of isocyanoacetate with o-carborane-containing aldehyde to afford oxazoline, a key intermediate of this route. Moreover, syntheses of CMHT-delivatives are currently progress.

We would like to report the synthesis of these boron compounds and biological properties toward tumor cells.

Straightforward synthesis of *N*-hydroxy peptides

Pascal Maire, Véronique Blandin, Monique Lopez, and **Yannick Vallée**

LEDSS, UMR CNRS-Université Joseph Fourier, BP 53, 38041 Grenoble Cedex 9, France

The *N*-hydroxy amide link presents interesting features. For instance, the presence of the hydroxyl was shown to enhance the anti-HIV activity of designed peptidic molecules thanks to supplementary hydrogen bonding with the receptor, or to induce conformational changes in peptides. Furthermore the strong iron complexation properties of the hydroxamate moiety was used for the design of good siderophores.

The obvious method for the synthesis of *N*-hydroxy peptides would be the direct coupling of a *N*-hydroxy amino acid with an activated peptide residue. However products resulting from *O*-acylation as well as from *N*-acylation can be obtained. Protection of the OH group as a benzyl ether has thus been used to overcome this problem. This requires an additional deprotection step but above all *N*-benzyloxy amino acids show a decreased reactivity in coupling reactions when bulky residues are at stake.

The poster will present our recent results on a straightforward sequence allowing the preparation of various *N*-hydroxy peptides. The *N*-hydroxy amino functionality is obtained via oxime reduction. Conditions for selective *N*-acylation will be discussed. Once the *N*-hydroxy amide link is formed, the pseudopeptide can be further elongated via classical peptide synthesis. This gives in particular an easy access to peptides presenting several hydroxamate motifs and thus several potential chelating sites.

Synthesis of polypeptide antibiotics emerimicin III and (Aib[12])-emerimicin

H. Brückner and **M. Currle**

Interdisciplinary Research Center, Department of Food Sciences, University of Giessen, Heinrich-Buff-Ring 26, D-35392 Giessen, Germany

The 15-residue polypeptide antibiotic emerimicin III, a member of the membrane active 'peptaibol' family, and the (Aib[12])-analogue of the structures Ac-Phe-Aib-Aib-Aib-Val-Gly-Leu-Aib-Aib-Hyp-Gln-D-Iva (Aib[12])-Hyp-Ala-Pheol (Ac, acetyl; Aib, alpha-aminoisobutyric acid; Hyp, *trans*-L-hydroxyproline; Iva, isovaline; pheol, L-phenylalaninol) were synthesized by stepwise conventional solution phase synthesis using the Z-O*t*Bu(OMe) strategy and HOBt/EDC as coupling reagents. The (Aib[12])-emerimicin III was synthesized in order to explore the synthetic route to emerimicin III.

The protected non-protein amino acid Z-D-Iva, required for the synthesis of natural emerimicin III, was obtained from ClAc-DL-Iva-OH (ClAc, monochloroacetyl) by stereospecific cleavage of the racemate with hog kidney acylase, removal of the L-Iva liberated by cation exchange, and acidic hydrolysis of the remaining ClAc-D-Iva-OH. The resulting D-Iva was converted into Z-D-Iva by treatment with Z-Cl.

Intermediates resulting from the syntheses were fully characterized and the identity of the synthetic peptide with the natural emerimicin III was proven by mass spectrometry, HPLC and antibiotic activity towards *Micrococcus luteus*.

New building blocks for peptide modification

Klaus Burger[1], **Gabor Radics**[1], **Ksenia Pumpor**[1], **Jan Spengler**[1], and **S. M. El-Kousy**[2]

[1] Organisch-Chemisches Institut der Universität Leipzig, Johannisallee 29, 04103 Leipzig, Germany
[2] Faculty of Science, Minufiya University, Shebin El-Kom, Egypt

The number of reports focusing on peptidomimetics built from two or more different types of monomers is still growing. Consequently, the development of new methodology for the synthesis and the incorporation of monomers like α-, β- and γ-functionalized carboxylic acids (amino, hydroxy and mercapto acids) is of current interest.

We demonstrated that a new protection/activation concept, developed for regioselective functionalization of multifunctional α-amino acids like Asp, can also be applied to their α-hydroxy and α-mercapto analogues. The syntheses of L-isoserine and D,L-isocysteine from malic and thiomalic acid, represent the first examples of the application of this new approach to multifunctional α-hydroxy and α-mercapto acids. We now report on the synthesis of L-homoisoserine, α-methyl-homoisoserine and homoisocysteine and their incorporation into peptidomimetics using the ''hexafluoroacetone route''.

The use of ω-amino acids in peptide design is a newly emerging area of current research. This is mainly due to the ability of these amino acids to modify the geometry of the peptide backbone providing proteolitical resistance to bioactive peptide sequences.

α-Hydroxy acids besides α-amino acids and carbohydrates, belong to the most important and abundant representatives of low molecular compounds of the naturally occurring chiral pool, while mercapto carboxylic acids are rare. They are constituents of natural products, for example of biologically active natural peptides. However, mercapto acids as well as acylmercapto acids are interesting building blocks for medicinal chemistry. A β-mercapto acid is a subunit of *Captopril*. An α-mercapto acid subunit is present in *Omapatrilat* and *Gemopatrilat*, both are vasopeptidase inhibitors which are currently under clinical evaluation. Compounds containing the mercapto or the mercaptoacyl moiety often exhibit strong inhibitory effects on metal-containing enzymes.

New types of glycosylated amino acids, interesting candidates for drug design

Christoph Böttcher, Alois Haas, and **Klaus Burger**

Organisch-Chemisches Institut der Universität Leipzig, Johannisallee 29, D-04103 Leipzig, Germany
Ruhr-Universität Bochum, FNO 034, D-44780 Bochum; new address: Medonstraße 17, D-14362 Kleinmachnow/Berlin, Germany

Glycoproteins are ubiquous in nature, playing a key role in various recognition processes on membranes. The peptide and the carbohydrate part are linked together by glycosidic bonds. These bonds are potentially acid sensitive and in some cases labile even under moderate basic conditions. Furthermore, glycoconjugates isolated from biological sources or produced gentechnologically are microheterogeneous. The different properties exhibited by each component within these microheterogeneous mixtures create problems in determining the exact function through structure/activity relationships. Therefore, the development of new methodology for the assembly of more robust, homogeneous glycopeptide mimetics as well as the construction of new binding motifs by chemoselective ligation is of current interest.

The stepwise construction of glycosylated peptides from glycosylated amino acids or glycosylated small peptides is still the preferred strategy because enzymatic and solid phase techniques can be combined with chemistry in solution. The application of hexafluoroacetone as protecting and activating reagent offers a new, efficient access to *O*-, *N*- and *C*-linked glycoconjugates, like *N*-glycosylated malic and citramalic acid as well as *O*-glycosylated, *N*-glycosylated and *O*-, *N*-diglycosylated isoserine and homoisoserine derivatives. The isoserine and the homoisoserine derivatives represent glycosylated β- and γ-amino acids, respectively. Furthermore, ''the hexafluoroacetone strategy'' provides access to N-glycosylated tripeptoids in five steps starting from iminodiacetic acid.

The new protection/activation strategy offers preparative simple routes to new types of glycoconjugates which represent valuable building blocks for drug design.

Synthesis of modular dipeptide mimetics based on diazabicycloalkane backbones

Daniel C. Grohs and **Wolfgang Maison**

University of Hamburg, Institute of Organic Chemistry, Martin-Luther-King-Platz 6, 20146 Hamburg, Germany

The aim of the presented project is the synthesis of new dipeptide mimetics based on diazabicycloalkane backbones. Various azabicycloalkane systems have been used as rigid dipeptide mimetics for the determination of structure-activity relationships of bioactive peptides and as turn mimetics. Incontrast, diazabicycloalkanes have not been studied in detail, presumably due to the lack of attractive synthetic routes. Nevertheless diazabicycloalkanes are interesting ligands for proteases due to their relatively extended dipeptide backbone which also makes them interesting scaffolds for combinatorial chemistry in terms of general screening methods. We would like to present a short and efficient synthetic route to enantiomerically pure diazabicycloalkanes via *aza*-Diels-Alder reaction and a subsequent tandem sequence of oxidative cleavage and intramolecular ring closure. Our new synthetic route leads to enantiomerically pure products within five steps starting from readily available educts and allows the variation of both amino acid side chains of the dipeptide mimetics. Furthermore, the stereochemistry and ring sizes may be altered allowing a fine-tuning of dipeptide backbone

conformations. Linker moieties can be attached to different positions of the diazabicycloalkane scaffold providing conjugation sites to other functional molecules such as markers or cytostatica. Diazabicycloalkanes might thus proof useful as modular ligands for cancer specific receptors.

Synthesis of amino acids with methyleneoxy bond: application in peptide analogs

J. Hlaváček and **J. Mařík**

Institute of Organic Chemistry and Biochemistry, Academy of Sciences, 166 10 Prague 6, Czech Republic

Isosteric replacement of peptide bond can lead to metabolically stable peptidomimetics. Our interest was focused to methyleneoxy surrogate, which provides flexible and lipophilic structure with enhanced metabolic stability and resembles the geometry of the peptide bond in extended conformation. Because of the lack of H-bonding, it can influence a β-turn in corresponding peptides and change their conformation and interaction with receptor. We prepared pseudodipeptides H-Pro-ψ[CH_2O]Ala-OH (**1**), H-Tyr(Bzl)-ψ[CH_2O]-Asp(OtBu)-OH (**2**), H-Pro-ψ[CH_2O]D-Thr-OH (**3**), H-Tyr-ψ[CH_2O]Ile-OH (**4**), H-Tyr-ψ[CH_2O]Phe-OH (**5**), H-Phe-ψ[CH_2O]Phe-OH (**6**) and introduced them into molecules of biologically active peptides. Pseudodipeptide **1**, was prepared by acylation of the O-protected prolinol by (2*R*)-2-bromopropionic acid followed by cyclization to (3*S*, 6*S*)-2-oxo-3-methyl-l-aza-4-oxabicyclo-[4.3.0]nonan and acidic hydrolysis to open the lactam ring between the nitrogen and carbonyl group. In the synthesis of pseudodipeptides **2**–**6**, the (5*S*)-5-substituted morpholin-3-one ring was prepared first with the nitrogen atom protected by bulky Boc group. Dehydration has followed quenching the enolate with benzaldehyde (**2**, **3**) or 2-butanone (**4**). The double bond was hydrogenated with high stereospecific purity and the (2*S*, 5*S*)-2,5-alkylated morpholin-3-ones were hydrolyzed by 6 M HCl to open the rings and obtain compounds **2**–**4**. The C_2-alkylation by tert.butyl 2-bromoacetate or acetaldehyde afforded directly pseudodipeptides **5** or **6**. The pseudodipeptides were utilized in the syntheses of the insect oostatic peptides, neurohypophyseal hormone analogs and inhibitors of HIV-1 protease. The introduction of methyleneoxy structure as the flexible peptide bond surrogate has caused significant changes in biological potency of corresponding peptide analogs depending on the peptide sequence and the site of the substitution performed.

Non-natural amino acids as building blocks for gramicidin-hybrid ion channels

H.-D. Arndt[2], **D. Bockelmann**[3], **A. Knoll**[2], **S. Lamberth**[3], **C. Griesinger**[3], **U. Koert**[1], **J. Pfeifer**[1], **L. Al-Momani**[1], and **P. Reiß**[1]

[1] Fachbereich Chemie, Philipps-Universität Marburg, 35032 Marburg, Germany

[2] Institut für Chemie der Humboldt-Universität zu Berlin, Berlin-Adlershof, Germany

[3] Max-Planck-Institut für Biophysikalische Chemie, Göttingen, Germany

The channel-mediated transport of ions through a membrane is a biological key function. Here we report on progress in the synthesis, the functional as well as the structural analysis of gramicidin-hybrid channels. Important topics are ion selectivity, channel-dwell time, asymmetric incorporation and function in the phospholipid bilayer. Channel building blocks such as THF and THP amino acids as well as cyclohexylether amino acids were synthesized stereoselectively and incorporated into the gramicidin motif. The functional analysis of the gramicidin hybrids thus obtained was done by single-channel-current measurements in planar lipid bilayers and by patch clamp studies in trabecular meshwork cells. Structural studies were accomplished by NMR.

Synthesis and histone deacetylase inhibiting activity of 3-[4-(2-benzoylethenyl)-1-methyl-1*H*-2-pyrrolyl]-*N*-hydroxy-2-propenamides, a novel class of synthetic inhibitors highly selective for class II histone deacetylases

A. Mai[1], **S. Massa**[2], **I. Cerbara**[1], **S. Valente**[1], **P. Loidl**[3], and **G. Brosch**[3]

[1] Dipartimento di Studi Farmaceutici, Università degli Studi di Roma "La Sapienza", P. le A. Moro 5, 00185 Roma, Italy

[2] Dipartimento Farmaco Chimico Tecnologico, Università degli Studi di Siena, via A. Moro, 53100 Siena, Italy

[3] Department of Molecular Biology, University of Innsbruck, Fritz-Pregl-Strasse 3, 6020 Innsbruck, Austria

Histone deacetylase (HDAC) is a family of enzymes playing an important role in gene expression. Because it has been reported that its inhibition brought about cell-cycle arrest and induced apoptosis and/or differentiation, HDAC is considered a target for new types of pharmaceuticals.

Recently, we reported a new series of hydroxamic acid-containing compounds, namely 3-(4-aroyl-1*H*-2-pyrrolyl)-*N*-hydroxy-2-propenamides (APHAs), as a novel class of HDAC inhibitors. Our lead compound, 3-(4-benzoyl-1-methyl-1*H*-2-pyrrolyl)-*N*-hydroxy-2-propenamide (IC_{50} against maize HD2 = 3.8 microM), showed HDAC inhibitory activity both *in vitro* and *in vivo* (histone hyperacetylation induced on mouse A20 cells) and resulted endowed with antiproliferative and cellular differentiation activities in murine erytroleukemic cells. Starting from these findings we performed various chemical modifications on different portions of such lead, with the aim to define structure-activity relationships and to improve HDAC inhibitory activity. All newly synthesized derivatives were tested against maize HD2 and maize HD1-B and HD1-A, two deacetylase enzymes homologues of class I and class II mammalian HDACs, respectively. Interestingly, properly substituted 3-[4-(2-benzoylethenyl)-1*H*-2-pyrrolyl]-*N*-hydroxy-2-propenamides were not very potent against HD2, slightly active against HD1-B, and endowed with high inhibitory activity against HD1-A enzyme. IC_{50} values of such compounds against HDA-1 are in the range 50–400 nM, and the class II selectivity ratio is 30–180 depending on the type and the position of substituents on the benzene ring. Our 3-[4-(2-benzoylethenyl)-1*H*-2-pyrrolyl]-*N*-hydroxy-2-propenamides represent the first sample of class II selective HDAC inhibitors.

Efficient synthesis of structurally diverse aza- and diazabicycloalkanes: scaffolds for modular dipeptide mimetics with tunable backbone conformation

Wolfgang Maison

Universität Hamburg, Institut für Organische Chemie, Martin-Luther-King-Platz 6, 20146 Hamburg, Germany

Stereoselective synthesis of modular dipeptide mimetics based on an aza- and diazabicycloalkane scaffold are reported. The routes start from readily available enantiomerically pure azabicycloalkenes. Key steps of these sequences are tandem reactions consisting of an oxidative cleavage of bishydroxylated azabicycloalkane precursors and a subsequent intramolecular trapping of the resulting bisaldehyde. Depending on the intramolecular trapping reaction either azabicycloalkanes (C–C-bond formation) or diazabicycloalkanes (aminal formation) can be synthesized in a highly stereoselective manner. These highly functionalized and orthogonally protected heterocycles can be easily converted into a number of dipeptide mimetics with defined and variable stereochemistry and a number of different amino acid sidechains. Backbone dihedral angles within these dipeptide mimetics can be tuned by varying the stereochemistry and the ring sizes of the bicyclic scaffold. A set of conformationally constraint dipeptide analogues mimicking peptide backbone geometries ranging from extended to turn conformations is thus accessible in only

a few synthetic steps. Suitable linker moieties for conjugation of these scaffolds to other functional molecules like marker or solid phases are introduced making these compounds modular dipeptide mimetics (structures **A** and **B**). Applications of these structures in our laboratory include their use as modular ligands for cancer specific receptors and as solid phase attached scaffolds in combinatorial chemistry.

Critical residues in the activation of phenylalanine hydroxylase by L-phenylalanine

Aurora Martínez, Knut Teigen, and **Matthías Thórólfsson**

Department of Biochemistry and Molecular Biology, University of Bergen, Jonas Liesvei 91, 5009-Bergen, Norway

Phenylalanine hydroxylase is a multidomain tetrameric enzyme that is activated by its substrate L-phenylalanine and displays positive cooperativity for substrate binding. This cooperative response is believed to be of physiological significance as a mechanism that controls L-Phe homeostasis in blood. The binding of L-Phe to the enzyme is believed to result in the displacement of an N-terminal autoregulatory sequence that partially covers the active site. However, there is scarce information available on the allosteric mechanism and how the effects of this displacement are propagated to the other domains and subunits. In the unactivated enzyme, Tyr377 in the catalytic domain is hydrogen bonded to Ser23 at the autoregulatory inhibitory N-terminal sequence, and kinetic and spectroscopic investigations of site-directed mutants, as well as molecular dynamics simulations, indicate that this hydrogen bonding network is disturbed on activation by L-Phe. In addition, we have prepared mutants at positions Cys237 and Arg68 with substitutions of different charge and size, since these residues also seem to play an important role in the transmission of activating conformational changes. Accordingly, the mutations C237D, R68A and C237A cause an increase of the basal activity and affinity for L-Phe, while the mutation C237R results in reduced affinity for the substrate and elimination of the positive cooperativity. All together our results based on experimental and molecular modeling/computational approaches indicate that the activation of phenylalanine hydroxylase induces a series of conformational changes including both the displacement of the inhibitory N-terminal sequence (residues 19–33), domain movements around the loops 111–117 and 68–75 and reorientation of the tetramerization helices.

Microwave assisted synthesis of biologically active compounds

Firouz Matloubi Moghaddam

Sharif University of Technology, Department of Chemistry, P.O. Box 11365-9516 Tehran, Iran

Microwave activation as a non-conventional energy source has became a very popular and useful technology in organic chemistry. The number of annual publications on microwave assisted organic chemistry is growing rapidly with almost one thousand publications in print sice the pioneering work of Gedye in 1986. Most of these publications describe important accelerations for a wide range of organic reactions especially when carried out under solvent-free conditions. The combination of solvent-free reactions conditions and microwave irradiation leads to large reductions in reaction times, in selectivity with several advantages of the eco-friendly approach, termed green chemistry. Since 1995 our research group is actively working in this area and has applied the microwave dielectric heating for the promotion of various organic reactions such as the Michael addition of amines to the α,β-unsaturated carbony compounds (*synthesis of β-aminoacids*), Fries rearrangement, Thia friesRearrengement, Claisen rearrangement, β-Elemination of sulfoxides, Willgerodt-Kindler reaction, Isomerization reactions, phenols alkylation, Beckmann rearrangement, Leukart reaction, ...

A rapide Michael addion of amines to the α,β-unsaturated carbony compounds has been achieved in good to excellent yield for the synthesis of *β-aminoacids* in the presence of water under microwave irradiation. In the absence of water and under conventional methods, the reaction does not proceed or take place in very low yield after a long reaction time. The reaction time in this case is 2 minutes.

The cinnamyl esters of Various phenols and naphthols were transformed to the biologically active Flavanones in a one-pot Tandem Fries reaction conjugate addition under microwave irradiation in dry media. The reaction proceeded smoothly in 7 minutes with excellent yields and regioselective acyl migration to the ortho position.

Aldehydes, aryl alkyl ketones and styrenes were efficiently transformed to the thioamides with the same number of carbon atomes via Willgerodt-Kindler reaction under micrewave irradiation in solvent-free conditions. The prepared Thioamides or their corresponding carboxylic acides are very useful organic compounds which have been used as versatile intermediates for the synthesis of biologically heterocyclic compounds, pesticides, antiinflammatory, and antiulser agents.

Aryl alkyl ketones and schiff bases are efficiently transformed to the primary, secondary amines or *α-amino acides* by means of the Leukart reaction under microwave irradiation. The reaction proceeds in about 5 minutes and the yields are excellent. All aspects of these reactions will be discussed in this presentation.

New developments in the synthesis of fluorinated analogues of glutamic acid and glutamine

Patrick Meffre[1], **Rajesh Dave**[1], and **Bernard Badet**[2]

[1] ENSCP, Laboratoire de Synthèse Organique, 11 rue P. et M. Curie, 75231 Paris cedex 05, France

[2] ICSN-CNRS, Avenue de la terrasse bat 27, 91198 Gif sur Yvette, France

In living cells, glutamine represents one of the main storage forms of nitrogen and is a major physiological source of ammonia for the biosynthesis of aminoacids, aminosugars, purine and pyrimidine nucleotides and coenzymes.

Glutamine-dependent amidotransferases perform nitrogen transfer from the amide group of glutamine to various electrophiles. When the latter is fructose-6P, the product of the reaction catalysed by glucosamine-6P synthase is D-glucosamine 6-phosphate, a structural building block of peptidoglycan (bacteria) and of chitin and mannoproteins (fungi). The considerable involvement of glucosamine-6P synthase in a number of biological processes as well as its potential as a therapeutic target in a disease such as type-2 diabetes, shows that this enzyme should be understood at a molecular level.

Fluorinated analogues of glutamine are expected to interfere with this biological process due to the strong electron withdrawing effect of fluorine atom (without significant steric consequence), inducing modulation of binding and/or electronic properties. These compounds might therefore behave as reversible or irreversible active site-directed enzyme inhibitors.

Results in the synthesis of two fluorinated analogues of glutamine: L-4,4-difluoroglutamine and (*S*)-2-amino-5-oxo-6,6,6-trifluoro hexanoic acid, a trifluoromethyl ketone analogue of glutamine, will be discussed.

Synthesis of non-natural amino acids based on the ruthenium-catalyzed oxidation of a phenyl group to carboxylic acid

P. Moutevelis-Minakakis, Ch. Sinanoglou, V. Loukas, and **G. Kokotos**

Laboratory of Organic Chemistry, Department of Chemistry, University of Athens, Panepistimiopolis 15771 Athens, Greece

Non-proteinogenic amino acids play an important role in the design and synthesis of pharmacologically relevant molecules, peptide mimetics and

enzyme inhibitors. The interest for homologated amino acids is increasing, as a result of the findings that peptides constructed from β- and γ-amino acids can adopt helix, sheet or reverse turn conformations. In addition, β- and γ-peptides may be suitable for pharmaceutical applications because they are stable against common proteases. We present here a general methodology for the synthesis of β-, γ- and δ-amino acids with proteinogenic side chains starting from α-amino acids. Our strategy to synthesize homologated amino acids was based on the ruthenium-catalyzed oxidation of a phenyl group to carboxylic acid. Boc-Phe-ol and Boc-Leu-ol, prepared from the corresponding α-amino acids by the mixed anhydride-$NaBH_4$ method, were oxidized to the corresponding aldehydes by NaOCl in the presence of 4-acetamido-TEMPO. Wittig olefination of Boc-phenylalaninal with stabilized or non-stabilized ylides, followed by hydrogenation, produced saturated Boc-protected amines containing a phenyl group. The aromatic ring of these compounds was oxidized to carboxylic acid by $BuCl_3 \cdot XH_2O$ using $NaIO_4$ in a biphasic system (CCl_4, CH_3CN, H_2O) leading to a variety of β-amino acids. Boc-leucinal was treated with the ylides generated from $Br^- Ph_3P^+CH_2C_6H_5$ or $Br^- Ph_3P^+(CH2)_nC_6H_5$, and the products of the olefination were hydrogenated. Oxidation under the conditions described above led to γ- and δ-leucine. The route described for the synthesis of γ- and δ-amino acids permits the insertion of any chain length between the amino and carboxy funcionalities as a result of the original choice of the starting ylide chain length.

Design and synthesis of novel (*S*)-pyroglutamic acid based antihypertensive agents

P. Moutevelis-Minakakis[1], **C. G. Kokotos**[1], **P. Kontogianni**[1], and **T. Mavromoustakos**[2]

[1] University of Athens, Department of Chemistry, Laboratory of Organic Chemistry, Panepistimiopolis, Athens 15771, Greece
[2] Institute of Organic and Pharmaceutical Chemistry, National Hellenic Research Foundation, Athens, Greece

Hypertension is a risk factor that may cause severe health problems. AT1 antagonists constitute a new generation of drugs for the pressure regulation and are designed and synthesized to mimic the C-terminal segment of Angiotensin II (Ang II) and to block its binding action on AT1 resceptor. To explore the stereoelectronic requirements for drug activity, the conformational analysis of Ang II and its derivatives as well as the AT1 antagonists belonging to SARTANs class of molecules have been studied. Such studies led to the design and synthesis from (*S*)-pyroglutamic acid of (5*S*)-1-benzyl-5-(1H-imidazol-1-ylmethyl-)-2-pyrrolidinone, a novel structure with considerable bioactivity (71% compared to the drug losartan) which mimics the His^6-Pro^7-Phe^8 sequence of Ang II. Novel analogs which mimic Tyr(Ome)-His^6-Pro^7-Phe^8 part of competitive antagonist of Ang II, sarmesin, were synthesized as follows:

Methyl (*S*)-pyroglutamate was treated with NaH and subsequently with m-methoxy benzylbromide, and the product was reduced to the corresponding alcohol by $LiBH_4$. The hydroxy group of *N*-(m-methoxybenzyl)-(*S*)-pyroglutaminol was activated by conversion to tosylate and was reacted with sodium imidazole or lithium benzimidazole. The bioactivity of these new analogs is under investigation.

Discovery, synthesis and SAR of *β*-amino acid BAY 10-8888/PLD-118, a novel antifungal for treatment of yeast infections

J. Mittendorf[1], **F. Kunisch**[2], **M. Matzke**[1], **H.-C. Militzer**[2], **K.-M. Mohrs**[1], **A. Schmidt**[1], and **W. Schoenfeld**[3]

[1] Bayer AG, Pharma Research, Wuppertal, Germany
[2] Bayer AG, Central Research, Leverkusen, Germany
[3] PLIVA d.d., Research and Development, Zagreb, Croatia

Major increases in the incidence of systemic fungal infections caused by the yeast *Candida albicans* have been observed during the last two decades, particularly in immunocompromised patients. A critical need exists for new antifungal agents to treat these life-threatening infections.

1-Aminocyclohexane-2-carboxylic acids, which were originally designed at Bayer AG as pyridoxal phosphate suicide inhibitors, turned out to have also activity against *C. albicans*. Along with the reported antifungal activity of the natural β-amino acid Cispentacin this result prompted us to initiate a derivatization program to identify cyclic β-amino acids with superior efficacy and tolerability. More than thousand derivatives were synthesized and their structure-activity-relationship investigated. Among these compounds BAY 10-8888 ((−)-(1R,2*S*)-2-amino-4-methylene-cyclopentane carboxylic acid) exhibited an excellent biological profile with regard to *in vitro*, *in vivo* activity, PK, solubility and safety. The stereo- and regioisomers as well as many closely related analogs showed no or lower antifungal activity demonstrating strict structural requirements for antifungal activity within this class. BAY 10-8888 can be prepared in 7 steps using an highly enantioselective, quinine-mediated alcoholysis of a corresponding meso-anhydride in the key step. BAY 10-8888 has been licensed to PLIVA and is currently investigated in phase II clinical studies as PLD-118.

Stereoselective *aza*-Diels-Alder reactions of alkoxy- and aryloxycarbonylimino-acetic acid ester

Alexander H. G. P. Prenzel and **Wolfgang Maison**

Institute of Organic Chemistry, University of Hamburg, Germany

The widespread use of biologically active and medicinally important heterocyclic peptide mimetics based on an aza- or diazabicycloalkane scaffold has led to significant interest in the development of new processes which allow the preparation of these compounds in an efficient and stereoselective manner. Amongst the available methods for achieving this goal, *aza*-Diels-Alder processes represent a particularly direct and attractive technique to form these heterocyclic compounds in enantiomerically pure form with diverse stereochemistry. In particular, the application of chiral lewis acid catalysts to mediate these reactions provides an opportunity to exert further control over the efficiency and selectivity. We would like to present a short and efficient route to *in situ* formed alkoxy- and aryloxycarbonylimino-acetic acid ester as highly reactive precursors for the *aza*-Diels-Alder reaction and the development of a stereoselective cycloaddition to give protected azabicyclo[2.2.1]alkene amino acids that are important starting materials for further synthetic routes to biologically and pharmaceutically active compounds. Most of the known *aza*-Diels Alder protocols use *N*-alkyl, -aryl and -tosyl substituted imines as dienophils resulting in difficult deprotection of the *aza*-Diels-Alder products. Our use of alkoxy- and aryloxycarbonylimino-acetic acid ester as dienophils, in contrast, leads to the formation of carbamat protected (e.g. Boc, Cbz or Fmoc) azabicycloalkenes which can be easily deprotected by standard procedures.

Synthesis and properties of cluster rhenium compounds with amino acids as ligands

N. I. Shtemenko[1] and **A. V. Shtemenko**[2]

[1] Dniepropetrovsk National University, Board of Biophysics and Biochemistry, Dniepropetrovsk, Ukraine
[2] Ukrainian State University of Chemical Technology, Board of Inorganic Chemistry, Dniepropetrovsk, Ukraine

Cluster compounds of rhenium (III), which have different coordination of amino acids (AA) around binuclear comlexforming center – Re_2^{6+} – of types: $(AA)_4[Re_2Cl_8]Cl_2$, $(AA)_2[Re_2Cl_8] \cdot H_2O$, $(AA)_2[Re_2Cl_8]$ and $[Re_2(AA)_2Cl_5(H_2O)]Cl \cdot 2H_2O$ are synthesized and investigated. Free amino acids from plant and animal tissues are involved in

the process of complexation. Chemical properties and structure of obtained compounds are studying. Influence of fatty radical length in the amino acid ligand on week interaction between binuclear anion $[Re_2Cl_8]^{2-}$ and protonized amino acid are discussed. Role of hydrogen bonds in formation of crystal unit cell of investigated substances is shown. These two factors are the reason of formation of staggered conformation of an anion $[Re_2Cl_8]^{2-}$ in some substances together with existence of quadruple Re–Re bond. Some of synthesized compounds showed cell-stabilizing activity against osmotic hemolysis and ability to change the forms of cells. Tetrachlorodi-μ-(γ-aminobutirato)dirhenium(III) chloride showed: antitumour, antiradical and antioxidant properties in models *in vivo* and *in vitro*. It is shown that antioxidant properties of rhenium cluster compounds are connected with quadruple Re–Re bond and depends from position of an AA around cluster Re_2^{6+} fragment. Reaction between antigene (Ag – standart human blood serum) and antibody (Ab – antiserum against IgA, IgM, IgG) was studied in the presence of $[Re_2(GABA)_2Cl_5(H_2O)]Cl \cdot 2H_2O$. The most shifts in immunoprecipitation lines were noticed in experiments with IgG. Antigenic properties of the rhenium cluster compound may be explained by conformational shifts of proteins, which doesn't bring perturbation of complementarity of the antigene-antibody reactive sites.

Synthesis and antiplatelet effects of RGD peptides incorporating salicylic acid derivatives

Yiannis Sarigiannis[1], **George Stavropoulos**[1], **Maria Liakopoulou-Kyriakides**[2], and **Pantelis Makris**[3]

[1] Department of Chemistry, University of Patras, 265 04 Patras, Greece
[2] Department of Chemical Engineering, Aristotle University of Thessaloniki, 540 06 Thessaloniki, Greece
[3] Haemostasis and Thrombosis Unit, AHEPA Hospital, 540 06 Thessaloniki, Greece

Integrins constitute a large family of heterodimeric cell-surface transmembrane receptors, which play a major role in cell/cell and cell/matrix adhesive interactions. The Arg-Gly-Asp (RGD) sequence is known to be the integrin recognition site of many extracellular matrix proteins such as fibronectin, osteopontin, collagen, fibrinogen, von Willebrand factor etc. Because RGD-containing proteins and peptides have been shown to bind to the platelet glycoprotein receptor Gp IIb/IIIa, several linear and cyclic RGD-containing peptides have been modeled to inhibit platelet aggregation. On the other hand, it is well known that low doses of aspirin (acetyl salicylic acid) decrease platelet aggregation by causing an inhibitory effect on thromboxane A_2 production by platelets.

We have already reported that the combination in the same molecule of dipeptide or tripeptide amides, containing amino acid derivatives of RGD sequence and salicylic acid derivatives at their N-terminal amino group, have shown satisfactory effect against human platelet aggregation *in vitro* and important specificity for the Gp Ib receptor. In an attempt to synthesize more potent inhibitors of platelet aggregation, we tried out the synthesis of RGD analogs incorporating thiosalicylic acid, 5-methyl salicylic acid, 5-amino salicylic acid, 2-methoxy benzoic acid, etc. The synthesized compounds, mainly by solid phase peptide synthesis, were purified by RP-HPLC, lyophilised and identified by FT-IR, H^1- NMR and ESI-MS spectra. The compounds were tested for inhibitory activity on human platelet aggregation *in vitro*, by adding common aggregation reagents (collagen, ADP, thrombin) to citrated platelet rich plasma (PRP). The aggregation was determined using a dual channel electronic aggregometer by recording the increase of light transmission. In order to get more information about the compounds' specificity for the Gp receptors we used flow cytometry with monoclonal antibodies against Gp Ib, Gp IIb/IIIa, Gp IIIa and GMP140 receptors. The IC_{50} values of the synthesized and tested compounds and the flow cytometry results will be discussed in details.

Synthesis of substance P (SP) analogs incorporating D-Trp and peptoid-peptide hybrids. Study of their antiproliferative properties *in vitro*

P. Vakalopoulou[1], **G. Stavropoulos**[1], **T. Kalemi**[2], and **G. Galactidou**[2]

[1] Department of Chemistry, University of Patras, 265 00 Patras, Greece
[2] Department of Research Theagenion Cancer Institute, 540 07 Thessaloniki, Greece

Synthetic peptides are currently under investigation as possible antitumor agents. The Substance P (SP) analog [D-Arg[1], D-Phe[5], D-Trp[7,9], Leu[11]]SP (antagonist D) and also the C-terminal analog [Arg[6], D-Trp[7,9], MePhe[8]]SP_{6-11} (antagonist G) inhibit the tumor growth and cell proliferation of Small Cell Lung Cancer (SCLC) *in vitro* and *in vivo*. The antagonists D and G have also been found to inhibit the DNA synthesis and the proliferation of Prostate Cancer cell lines.

In the present study a series of tetra- and tri-peptide analogs have been synthesized, based on the sequence of antagonist G using the stepwise synthesis or the fragment condensation method, either in solution or in Solid Phase Peptide Synthesis. All the synthesized analogs were purified (HPLC) and identified (ESI-MS, ^{1}H-NMR, FT-IR).

Some of the analogs are peptides incorporating in their chain D-Trp, whereas some others are peptoid-peptide hybrids, which are oligomeric peptidomimetics containing one or more N-substituted glycine residues. The incorporation of N-substituted glycine in the peptide chain is expected to improve their stability against proteases. Thus the peptoid-peptide hybrids have incorporated the monomer [N(CH_2-Ph)-CH_2-CO-](NPhe) instead of the amino acid residue of [HN-CH(CH_2-Ph)-CO-](Phe).

Subsequently the analogs were tested for their antineoplastic properties in several cancer cell lines. The peptide analog Arg-D-Trp-MePhe-D-Trp-OH showed antiproliferative activity for the cancer cell lines OAW-42 (human ovarian cancer) and T47D (breast cancer), leaving 67% survival fraction at the concentration of 100 μM. More results concerning the activity of other peptides, as well as the activity of peptoids, will be presented during the conference.

Synthesis and evaluation of 5-fluorodeoxyuridine derivatives possessing tumor-homing peptide unit

Zhouen Zhang, Kazuhito Tanabe, Hiroshi Hatta, and **Sei-ichi Nishimoto**

Department of Energy and Hydrocarbon Chemistry, Faculty of Engineering, Kyoto University, Kyoto 606-8501, Japan

5-Fluorodeoxyuridine (5-FdUrd) is a well-known antitumor agent. Since the clinical application of 5-FdUrd is limited because of its high toxicity to the normal cell as well as the tumor cell, the development of 5-FdUrd prodrugs has received much attention.

The cyclic peptide Cys-Asn-Gly-Arg-Cys (CNGRC) is recognized as a tumor-homing peptide that can selectively bind to tumor by a specific isoform of aminopeptidase (CD13) expressing in the angiogenic endothelial cells. We designed and synthesized 5-FdUrd derivatives tethered to peptide CNGRC by alkyl linker (5-FdUrd–$(CH_2)_n$–CNGRC) as tumor-homing prodrugs of 5-FdUrd. These prodrugs released 5-FdUrd via hydrolysis in both neutral aqueous media and fetal bovine

serum. While the prodrug **1** possessing propyl linker (n = 3) decomposed quickly with the half-life time of 2 h as the result of free 5-FdUrd release, the prodrug **2** possessing ethyl linker (n = 2) decomposed slowly with the half-life time of 72 h. These results indicate that the length of alkyl linker affect the stability of these prodrugs. Thus, these 5-FdUrd-oligopeptide conjugates would be prototype compounds for developing a new class of tumor-homing prodrugs of 5-FdUrd in the treatment of tumor.

Amino acids of erythrocytes and plasma under some diseases

E. A. Zabitskaya, O. O. Sorochan, and **N. I. Shtemenko**

Dniepropetrovsk National University, Dniepropetrovsk 49050, Ukraine

The qualitative and quantitative composition of free amino acids in blood was studied in healthy volunteers (control) and patients with anemia (vitamin B_{12} – 118 pg/ml, hemoglobine – 100 g/l). The content of free amino acids was determined in plasma and hetmolysates of red blood cells and the ratio between general pool of amino acids in plasma: in cell (PC) was calculated too. It was shown that general pool of amino acids was considerably increased as in plasma, as in erythrocytes in patients with anemia if been compared with control. There were Asp, Glu, Pro, Gly, Ser that prevailed in cell and Ser, Gly, Ala, Pro – in plasma. It was observed that the PC had essential changes in patients with anemia (5:1) whereas PC in healthy volunteers was (9:1). It is supposed that the obtained results confirm the protein destructive processes in tissues of organism as a consequence of vitamin B_{12} deficiency. Probably the evolution of anemia is accompanying with disturbances in the process of exchange of amino acids between plasma and a red blood cell. Thus PC may have a diagnostic role in some diseases connected with erythrocyte membrane changings.

RGDS peptide inhibits endothelial cells chemotaxis and induces caspases activation

M. S. Aguzzi[1], **C. Giampietri**[2], **F. De Marchis**[1], **R. Gaeta**[3,4], **M. C. Capogrossi**[1], and **A. Facchiano**[1]

[1] Lab. Patologia Vascolare, IDI, Rome, Italy
[2] Dip. Istologia ed Embriologia Medica, Univ. La Sapienza, Rome, Italy
[3] Divisione di Cardiochirurgia, IRCCS ''San Matteo'', Pavia, Italy
[4] Cattedra di Cardiochirurgia, Università di Messina, Messina, Italy

The RGD-motif present within a variety of extracellular matrix (ECM) proteins exhibits numerous biological effects, mainly related to the inhibition of integrin signaling. Peptides containing the RGD sequence exert pro-apoptotic, anti-coagulant and anti-metastatic activities, due to the anti-adhesive action on target cells. Since RGD has recently shown pro-apoptotic effect independent from its anti-adhesive action, we examined the effects of an RGD-containing peptide (RGDS) on human umbilical vein endothelial cells (HUVEC) seeded onto collagen IV to minimize the anti-adhesive effects. In fact, cell adhesion to collagen IV is know to be RGD-independent. Under such conditions RGDS significantly inhibited FGF-2 induced HUVEC proliferation and chemotaxis ($50 \pm 4\%$ inhibition, and $81 \pm 9\%$ inhibition, respectively at 500 μg/ml). Pre-incubation with caspases inhibitors (Z-VAD-FMK, Z-IEDT-FMK and Z-LEHD-FMK) strongly reduced the anti-chemotactic action, suggesting that caspase 8 and caspase 9 activation mediates the observed anti-chemotactic effect. Further experiments supported this hypothesis showing that RGDS-treatment significantly activated caspase 8 and caspase 9 after 4 h treatment and caspase 3 after 24 h by about two fold, leading to apoptosis. Finally, it was found that RGDS directly interacted with immobilized cytoplasmic extracts and with immobilized caspase 8 and caspase 9, suggesting that the direct interaction might induce the observed caspases activation.

These results indicate for the first time that RGDS directly binds and activates caspases 8 and 9 and, likely as consequence of this, inhibits chemotaxis and induces apoptosis of HUVEC, in an anchorage-independent way.

Amino Acid Transport

Molecular and functional characterisation of the hepatoma glutamine ASCT transporter

C. Bungard

Department of Biochemistry, Medical School, University Walk, Bristol, England

In hepatocytes glutamine is used as a substrate for urea synthesis and gluconeogenesis. Glutamine is transported into hepatocytes via a Na^+ dependent system N transporter. This transporter is specific for glutamine, asparagine and histidine and tolerates Li^+ substitution.

In contrast, hepatoma cell lines use glutamine as a major energy source. It is essential to the hepatoma cell and is transported 15 fold faster. It appears that transformed liver cells almost abolish system N expression and switch to an alternate transporter family known as system ASCT. This is also Na^+ dependent however it does not tolerate Li^+ substitution and shows much broader substrate specificity.

In rat hepatoma H4-II-E-C3 cell line a novel ASCT2 transporter has been cloned however it is not detected by PCR methods in normal rat liver. A different member of the ASCT family has also been identified and partially cloned in the human hepatoma cell line HepG2 and initial research indicates it is not expressed in normal human liver. It could therefore be possible to use this newly expressed glutamine transporter as a tumour marker of hepatoma cells.

Two approaches have been employed in order to elucidate the relationship between hepatoma cell growth and the ASCT2 expression. The first is 'knock-out' or 'knock-down' of the ASCT2 using antisense techniques. The second is to manipulate the growth rate of the cells and study the effect on ASCT2 expression. Initial studies show it is possible to alter the growth rate of the cells and this effects the ASCT2 expression.

Membrane transport of asparagine-synthase substrates in asparaginase-sensitive and resistant sarcoma cells

Bianca Maria Rotoli, Valeria Dall'Asta, Gian C. Gazzola, and **Ovidio Bussolati**

Department of Experimental Medicine, Unit of General and Clinical Pathology, University of Parma, Parma, Italy

Asparaginase (ASNase) causes an acute deprivation of intracellular asparagine and glutamine and the rapid induction of apoptosis in leukemia cells endowed with low levels of expression/activity of asparagine synthase (AS). However, in those cells no expression of sodium dependent aspartate transport has also been ascertained. To assess ASNase effects in solid tumor models, we have cultured Jensen sarcoma cells, a rat cell line extremely sensitive to ASNase, with increasing (from 0.005 to 5 U/ml) concentrations of the enzyme and obtained several clones of

resistant variants. In one of these clones (R1C8) AS activity is doubled compared with parental cells and further enhanced in the presence of the antitumor enzyme. Determinations of transport activities for aspartate, glutamate, and glutamine, the immediate and remote substrates for AS, demonstrate that the activity of EAAT-type transporters is barely detectable either in parental or resistant cells, both in the presence or in the absence of ASNase. On the contrary, both systems x_C^- (sodium-independent transport of anionic amino acids) and A (sodium dependent transport of neutral amino acids) are significantly stimulated in cells maintained in the presence of ASNase and are also induced by the enzyme acutely added to the culture medium. Transport activity of system ASC is lowered in resistant cells and scarcely influenced by enzyme treatment. These results suggest that low/absent expression of sodium-dependent transport systems for aspartate may represent a prerequisite for ASNase sensitivity and point to the induction of coordinate changes in membrane transport and intracellular enzyme activities by ASNase treatment.

Structural and functional studies of the bacterial protein-translocation complex

I. Collinson

Max Planck Institut für Biophysik, Frankfurt am Main, Germany

The Sec protein complex is localised in biological membranes to provide an essential and ubiquitous route for the translocation of secretory and membrane proteins. In bacteria, this transporter is formed by oligomers of a heterotrimeric membrane protein complex consisting of subunits SecY, SecE, and SecG. Translocating proteins are driven through the complex by an ATPase SecA or during their synthesis from bound ribosomes. The structure of the *E. coli* SecYEG assembly has been determined at 8 Å resolution (Breyton et al., 2002). The three-dimensional map calculated from two-dimensional SecYEG crystals reveals dimers of SecYEG within a phospholipid bilayer. This may represent the closed state of the protein-conducting complex. This dimer has been found to associate with translocating polypeptide (Bessonneau et al., 2002) and also with both monomeric and dimeric forms of the partner protein SecA. Complexes of the core complex together with peptides, SecA or with ribosomes are under current investigation.

Transduction pathways involved in the regulation of arginine transport in human endothelial cells

Rossana Visigalli, Roberto Sala, Amelia Barilli, Ovidio Bussolati, Gian C. Gazzola, and **Valeria Dall'Asta**

Dip. di Medicina Sperimentale, Sezione di Patologia Generale e Clinica, Università degli Studi di Parma, 43100 Parma, Italy

The regulation of arginine transport has been studied in human saphenous vein endothelial cells (HSVECs). Neither interferon-γ nor interleukin 1 modify the influx of the cationic amino acid. Conversely, tumor necrosis factor-α (TNF-α) and bacterial lipopolysaccharide (LPS) induce a transient stimulation of arginine transport through system y+, whereas system y + L activity is not affected by any compound. The enhancement of system y+ transport activity induced by TNF-α is referable to an increased expression of SLC7A2/CAT2B gene while SLC7A1/CAT1 expression is not altered by the cytokine. The suppression of PKC-dependent transduction pathways, obtained either with the inhibitor cheleritryne or with a chronic treatment with phorbol esters, does not prevent TNF-α effect on arginine transport. Also ERK and p38 MAP kinases do not appear to be involved in the cytokine effect, since arginine transport stimulation is unaffected by the inhibitors PD98059 and SB203580. On the contrary, several inhibitors of NF-κB pathway hinder both the induction of CAT-2B expression and the stimulation of arginine uptake. Wortmannin and rapamycin, inhibitors, respectively, of PI3 and mTOR kinases, do not prevent TNF-α-induced stimulation of arginine transport but stimulate arginine transport either in the absence or in the presence of the cytokine. However, only wortmannin significantly induces CAT2B expression. These results indicate that the activation of NF-κB pathway mediates the effects of TNF-α on SLC7A2/CAT2B expression and point to endothelial arginine transport as a target of multiple and overlapping regulatory mechanisms.

Pept2 knockout mice show altered renal handling of peptides

I. Frey[1], **I. Rubio-Aliaga**[1], **H. M. Eichinger**[2], **A. Drobyshev**[3], **J. Beckers**[3], and **H. Daniel**[1]

[1] Molecular Nutrition Unit, Technical University of Munich, Germany
[2] Laboratory of Bioanalysis, Technical University of Munich, Germany
[3] Institute of Experimental Genetics, GSF-National Research Center for Environment and Health, Germany

Transporters for di- and tripeptides that belong to the proton-coupled oligopeptide transporter family (POT) are found in prokaryotes and eukaryotes. In mammals two different systems have been identified. PEPT1 is mainly expressed in the small intestine where it mediates the absorption of dietary di- and tripeptides. PEPT2 on the other hand shows expression in various tissues. The highest PEPT2 expression is found in the kidney, where it mediates the reabsorption of filtered di- and tripeptides. To study the physiological role of PEPT2 we have generated a knockout mouseline that lacks a functional PEPT2 protein. The *Pept2*$^{-/-}$ mice showed markedly reduced accumulation of labelled model dipeptides in the kidney. Compensatory upregulation of PEPT1 and of the peptide histidine transporter PHT1 could not be observed. DNA microarray analysis revealed, that of amino acid transporters only 4F2hc is upregulated in the knockout mice. To determine dipeptides in urine amino acids in 24 h urine samples were analysed previous to and after digestion with a renal dipeptidase (EC 3.4.19.13). Previous to digestion there was a tendency towards higher amino acid excretion in the knockout mice. Dipeptidase digestion lead to a significant rise of the concentration of glycine and cystine in the *Pept2*$^{-/-}$ mice. This effect became much more distinct when mice were kept on a high protein diet. Moreover excretion of glycine, cystine, cystathionine, methionine, leucine, γ-amino butyric acid and ornithine was significantly elevated in the knockout-mice fed 30% protein. Our studies confirm the importance of PEPT2 in renal reabsorption of peptides.

3D-QSAR of peptide substrates of the mammalian peptide transporter PepT1

Sabine Gebauer[1], **Annegret Biegel**[1], **Ilka Knütter**[1], **Matthias Brandsch**[2], **Iris Thondorf**[1], and **Klaus Neubert**[1]

[1] Institute of Biochemistry,
[2] Biozentrum, Martin-Luther-University Halle-Wittenberg, Kurt-Mothes-Str. 3, D-06099 Halle/Saale, Germany

The mammalian peptide transporter PepT1 is a transmembrane protein that transports di- and tripeptides from the luminal membrane of mammalian intestinal cells into the blood. The system is driven by a transmembrane proton gradient and shows a broad substrate specificity. Besides small peptides also peptidomimetics and prodrugs such as β-lactam antibiotics, valacyclovir, δ-aminolevulinic acid, ACE inhibitors, bestatin and others are recognized and transported. These findings open the route to utilize the PepT1 pathway for oral drug delivery either by adaptation of the drug's structure to the requirements of the transporter or by conjugation of drugs with small and rigid substrates.

The very nature of membrane proteins has made it difficult to determine the structure of PepT1. Very little is known about either the active binding site of PepT1 or the bioactive conformation of its substrates. Work to date has focussed on an extensive measurement of substrate

affinities and functional analysis of chimeric mammalian peptide transporters. Molecular modelling approaches using substrate affinity information are an additional promising tool to study structural requirements for substrates. Computational studies published so far have been mainly directed toward the relatively rigid ß-lactam antibiotics and ACE inhibitors. Recently, a model has been proposed suggesting four key recognition elements.

In this contribution, we present a 3D QSAR model which is able to explain and predict affinities of structurally diverse substrates. On the basis of spatial arrangements of various field properties such as steric, electrostatic, lipophilic and hydrogen-bonding it is now possible to design novel substrates and inhibitors and predict their affinities.

Amino acid transport by the novel transporter rat PAT2

D. J. Kennedy and **D. T. Thwaites**

School of Cell and Molecular Biosciences, The Medical School, Framlington Place, University of Newcastle upon Tyne, Newcastle upon Tyne, NE2 4HH, UK

The H^+-coupled amino acid transporter PAT1 (proton-coupled amino-acid transporter 1) has been cloned from rat, mouse and human and a related transporter (PAT2) has been cloned from mouse and rat. PAT1 is expressed ubiquitously at the mRNA level and has been identified functionally at the small intestinal brush-border membrane. PAT2 expression is restricted to heart, brain, spleen, lung, skeletal muscle and kidney with the physiological role remaining unknown. Here we compare the function of PAT1 and rat PAT2. Both PAT1 and PAT2 function in a pH-dependent, Na^+-independent manner. The two PAT isoforms share a number of substrates such as proline, MeAIB, glycine and β-alanine with PAT2 being a high affinity transporter, compared to PAT1. There are, however, differences in substrate specificities between the two PAT isoforms with some substrates for PAT1 (e.g. GABA and taurine) being excluded by PAT2. In general PAT1 has a higher affinity for D-isomers with PAT2 having a lower affinity e.g. PAT1 has a higher affinity for D-serine than L-serine, whereas, PAT2 has a similar affinity for both D- and L-serine. PAT1 has similar affinity for both D- and L-alanine, while PAT2 has a greater affinity for L-alanine. The GABA analogues D- and L-pipecolic acid are also transported differently between the PAT clones with PAT1 having higher affinity for the D-isoform and PAT2 having a higher affinity for the L-isoform. In conclusion, PAT1 and PAT2 function in a similar manner albeit with differences in substrate specificity.

The role of CAT-transporter expression in human skin

Oliver Schnorr, Schristoph Suschek, and **Victoria Kolb-Bachofen**

Research Group Immunobiology, Med. Fac, Heinrich-Heine-University, Duesseldorf, Germany

Inducible nitric oxide synthase (iNOS) and arginase activities are important players in human skin epidermal function. For proper enzyme function the substrate availability of L-arginine for both enzymes and thus its transport across the cell membrane via the cationic amino acid transporters (CAT) is critical.

Here, we examine the expression of CATs and their functional role in modulating iNOS and arginase activities in human skin and primary keratinocytes, fibroblasts and endothelial cells as well as their impact on keratinocyte proliferation. Skin biopsies were found to constitutively express both CAT-1 and CAT-2 mRNA, an expression pattern known to occur in hepatocytes and muscle cells only. Next, we analysed the expression patterns in the different human skin cell types *in vitro*, i.e. in fibroblasts, dermal endothelial cells and keratinocytes as well as in the HaCat cell line. Ubiquitous CAT-1 mRNA expression was found in all cells, whereas constitutive CAT-2 mRNA expression occurs in resident keratinocytes and dermal endothelial cells only. *De novo* induction of CAT-2 and iNOS by proinflammatory cytokines was seen in fibroblasts and HaCat. Competitive inhibition of CAT-mediated L-arginine transport by culturing primary human keratinocytes in the presence of increased L-lysine concentration led to decreased iNOS and arginase activities with a concomitant significant decrease in keratinocyte proliferation. In summary, our results demonstrate for the first time that human keratinocytes constitutively express cationic amino acid transporters-1 and -2 and that CAT mediated L-arginine influx, is essential for both, iNOS and arginase enzyme activities, which in turn modulate proliferation and differentiation of human epidermal skin cells.

New aspects of substrate specificity for peptide transporters

Frederick H. Leibach[1], **Bianka Hartrodt**[2], **Ilka Knütter**[3], **Matthias Brandsch**[3], and **Klaus Neubert**[2]

[1] Department of Biochemistry and Molecular Biology, Medical College of Georgia, Augusta, Georgia, GA 30912-2100, USA
[2] Institute of Biochemistry, Department of Biochemistry/Biotechnology, and
[3] Membrane Transport Group, Biozentrum, Martin-Luther-University Halle-Wittenberg, D-06120 Halle, Germany

The existence of peptide transport systems for di- and tripeptides has been known for a long time but not always appreciated by the scientific community. It is now well established and generally accepted that a larger fraction of amino acids is taken up in the gastrointestinal tract in the form of small peptides rather than free amino acids. Estimates suggest between 30 and 70 per cent of a protein hydrolysate enter the intestinal epithelial cell in the form of peptides. Peptide transporters have very broad specificity and accept almost all possible di- and tripeptides derived from the 20 common amino acids. Interesting recent discoveries regarding substrate specificity show that they will also accept non-peptide substrates. Many publications have appeared in which the idea was tested that peptide transporters can be used to increase bioavailability of drugs. Assay systems employed in these studies range from intestinal tissue preparations, membrane vesicles, intestinal cell lines to heterologous expression of cloned peptide transporters. Many of these studies report kinetic constants for transport of putative prodrug substrates which may be questionable. We will present examples of peptide transporter substrates from the groups prodrugs, backbone-modified dipeptides, side chain modified dipeptides, natural di- and tripeptides, terminally blocked dipeptides, amino acid derivatives and others. We will suggest some guidelines and considerations for the characteristics of a substrate for peptide transporters.

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Inhibition of CAT2 transporter protects from immune-mediated diabetes, neurodegeneration and asthma

Carol L. MacLeod, Cathyryne K. Manner, Marc Rothenburg, and **Lesley Ellies**

University of California, San Diego, La Jolla, California, USA

CAT transporters are primary mediators of arginine flux in most mammalian cells. The Cationic Amino Acid Transporter family (*Cat*) of genes encode the y^+ transporter system that mediates arginine, lysine

and ornithine flux across cell membranes. Three known *Cat* transporter genes individually encode related, but distinct proteins. We took a genetic approach to identify the physiological and potential pathological function of the *Cat2* transporter, paying particular attention to the possible role of CAT2 in regulating nitric oxide production by inducible nitric oxide synthase (NOS2). Genetic ablation of *Cat2* provided the essential tool to characterize the role of this transporter in the regulation of NO production *in vitro* and the development and progression of mouse models of diabetes, asthma and multiple sclerosis. NOS2-mediated NO production assessed in macrophages, astrocytes and fibroblasts indicated that cells differ in their dependence on CAT2 for NO production and that the phenotype of $Cat2^{-/-}$ mice may not be predictable from the results of experiments with $Nos2^{-/-}$ mice. NO exhibits both protective and destructive effects in an experimental allergic encephalomyelitis, (EAE) animal model. Although *Nos2* deficiency exacerbated the clinical course of active EAE, *Cat2* deficiency delayed disease onset and reduced incidence. NO was reported to exacerbate clinical diabetes and it has well known inflammatory effects in asthma. Our data using *Cat2* deficient mice have documented a protective effect against experimentally induced diabetes and asthma. The precise mechanisms have not yet been elucidated but the data suggest that alterations in both adaptive and native immunity are implicated.

Nutritional and pharmacological manipulation of neutrophils (PMN): influence on markers of PMN immune function including intracellular taurine and taurine-dependent amino acids

J. Mühling[1], **M. Fuchs**[2], **J. Gonter**[1], **K. A. Nickolaus**[1], **M. E. Campos**[2], **D. Quandt**[1], **M. Müller**[1], and **G. Hempelmann**[1]

[1] Department of Anaesthesiology, Intensive Medicine and Pain Therapy, University Hospital, Justus-Liebig-University, Giessen, Germany

[2] Dr. Ing. Herbert Knauer GmbH, Berlin, Germany

In order to evaluate the influence of nutritional and pharmacological treatments on immune function markers in neutrophils (PMN) we studied the effects of taurine, β-alanine, several central and peripheral benzodiazepine agonists and antagonists (midazolam, flumazenil, Ro 5-4864, and PK 11195), as well as the glutamine analogue 6-diazo-5-oxo-norleucine (DON) on intracellular taurine and taurine-dependent amino acid content, as well as on important immune function markers (superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) formation, released myeloperoxidase (MPO) activity). Exogenous taurine (>0.5 mM) increased PMN intracellular taurine content whereas β-alanine, midazolam, Ro 5-4864 and DON affected a decrease. The peripheral benzodiazepine antagonist PK 11195 partially reversed the pharmacological effects of the benzodiazepine receptor ligands investigated (midazolam, Ro 5-4864). In PMN cells without an impaired metabolism nutritionally or pharmacologically induced changes in intracellular taurine content were accompanied by an inverse alteration in neutral amino acid concentrations, possibly representing a homeostatic reaction to changes in intracellular osmoregulation. Moreover, taurine decreased both O_2^- and H_2O_2 and increased MPO activity whereas β-alanine supplementation effected the reverse. We hypothesize that alterations in O_2^- and H_2O_2 formation may be due to either 1) changes in HOCl-mediated reactive oxygen species (ROS) caused by alterations in HOCl sequestration by taurine to form taurine chloramine or 2) changes in ROS-metabolism due to alterations in taurine-dependent MPO activity, or both. Harmful pharmacological stress induced by midazolam, Ro 5-4864 and DON causes maleficient intracellular conditions which adversely affect PMN function and negate the positive influence of intracellular taurine content in relation to PMN immune functionality.

Molecular physiology of the GABA cotransporter rGAT1

A. Peres[1], **S. Giovannardi**[1], **E. Bossi**[1], **F. Binda**[1], and **R. Fesce**[2]

[1] Laboratory of Cellular and Molecular Physiology, Department of Structural and Functional Biology, University of Insubria, Via Dunant 3, 21100 Varese, Italy

[2] Department of Neurosciences, S. Raffaele Institute, Via Olgettina 58, 20132 Milano, Italy

Electrophysiological determinations on cloned amino acid cotransporters overexpressed in heterologous systems have revealed that these membrane proteins possess different operating modes, depending on the presence or absence of the organic substrate. In addition to a transport-associated current, arising from the transmembrane charge transfer that accompanies amino acid uptake, most cotransporters exhibit, in the absence of organic substrate, transient currents elicited by sudden changes in membrane voltage or in co-ion concentration. Differently from the transmembrane transport-associated current, the transient current has the characteristics of an intramembrane charge displacement. Working on the neuronal GABA cotransporter rGAT1 expressed in Xenopus oocytes, we have observed that a simple relation links the kinetics of the intramembrane charge movement to the transmembrane transport current. That is, the amplitude of the latter at any voltage may be obtained by dividing the amount of charge displaced at the same voltage by the decay time constant of the former. This relation holds in a number of different experimental conditions, such as altered GABA and Na^+ concentrations, and also reduced Cl-concentrations. The system may be simulated by a simple three-state kinetic scheme in which GABA must bind after Na^+. The results suggest that GABA binding shifts the transporters from a capacitive-like behaviour to a conductive mode of operation, without strongly altering either the amount or the rate of charge movement. The partition between the two modes is governed by voltage and external Na^+ concentration and accounts for the observed voltage-dependence of the apparent GABA affinity of rGAT1.

PKC activation promotes the internalization of the human cationic amino acid transporter hCAT-1

Alexander Rotmann, Ursula Martiné, Dennis Strand, and **Ellen Ildicho Closs**

Department of Pharmacology, Johannes Gutenberg University, 55101 Mainz, Germany

The activity of the human cationic amino acid transporter hCAT-1 is decreased after protein kinase C (PKC) activation by phorbol-12-myristate-13-acetate (PMA). To address the question, if PKC directly phosphorylates hCAT-1, we removed all three PKC recognition sequences in hCAT-1 by mutating the respective serine residues (positions 476, 482 and 557) to alanine. The hCAT-1 mutant exhibited an unchanged L-arginine transport activity compared to wildtype hCAT-1 when expressed in *Xenopus laevis* oocytes. A 30 min treatment with 100 nM PMA lead to a 50% reduction in transport activity of both, mutant and wildtype hCAT-1. In addition, when stably overexpressed in human U373MG glioblastoma cells, the transport activity of mutant and wildtype hCAT-1 was downregulated to a similar extend after PKC activation. The inactive phorbol ester 4-α-PDD had no effect. Pretreatment with 1 μM bisindolylmaleimide I reduced the PMA effect significantly indicating that the reduction in transport activity by PMA was mediated by PKC. To study the subcellular distribution of the transporter, the enhanced green fluorescence protein (EGFP) was fused to the C-terminus of wildtype and mutant hCAT-1. Upon PMA treatment the membrane expression of both fusion proteins stably expressed in U373MG cells was reduced as shown by confocal microscopy. Western blot analysis of surface biotinylated membrane proteins showed a 20–30% decrease of

hCAT-1 in the membrane protein fraction after PMA treatment. Similar results were obtained when the cell surface expression of the endogenous hCAT-1 were studied in DLD-1 coloncarcinoma cells. Our results indicate an indirect inhibitory action of PKC on hCAT-1 activity and suggest that PKC activation promotes the internalization of hCAT-1 from the plasma membrane.

Structure/function relationship in the insect amino acid transporter KAAT1

V. Franca Sacchi[1], **Michela Castagna**[1], **Stefania A. Mari**[1], **Carla Perego**[1], **Andrea Soragna**[2], and **Elena Bossi**[2]

[1] Institute of General Physiology and Biological Chemistry, University of Milano, Via Trentacoste 2, 20134 Milano, Italy
[2] Department of Structural and Functional Biology, University of Insubria, Via Dunant 3, 21100 Varese, Italy

KAAT1 (K^+-dependent Amino Acid Transporter) is a neutral amino acid transporter, activated by K^+ or by Na^+ that was cloned from the midgut of *Manduca sexta* larva. The protein shows significant homology with members of the Na^+/Cl^--dependent neurotransmitter transporter super family. In this study we have analysed the physiological role of a number of amino acid residues by site-directed mutagenesis.

We have mutated residues highly conserved in the super family and residues that are typical of K^+-dependent transporters KAAT1 and CAATCH1. CAATCH1 has been cloned from *Manduca sexta* midgut and is 90% identical to KAAT1.

Some mutants, expressed in *Xenopus* oocytes, exhibit a modified leucine uptake. In some cases we have observed an activity recovery if the charge is conserved (E59D, D338E).

Interestingly the mutation of aspartate 338, a residue not conserved in the super family, seems to influence the cation selectivity of the cotransporter. D338EKAAT1 exhibits a reduced leucine uptake and reduced leucine induced current in the presence of sodium and a complete lack of leucine transport and leucine induced current in the presence of potassium, which is the physiological driver *in vivo*. In the absence of the organic substrate, large uncoupled currents have been measured. Possibly aspartate 338 contributes to the cation filter of the transporter.

Fate of elevated blood L-tryptophan after oral L-tryptophan administration in nephrotic rats

Emi Sasaki[1] and **Yoshiji Ohta**[2]

[1] Department of Biochemistry and
[2] Department of Chemistry, School of Medicine, Fujita Health University, Toyoake, Japan

We have reported that disappearance of elevated blood L-tryptophan after oral L-tryptophan administration is much faster in rats with puromycin aminonucleoside nephrosis than in non-nephrotic rats, and have suggested that this rapid disappearance of elevated blood L-tryptophan in nephrotic rats is mainly due to decreased serum albumin concentration. However, the fate of orally administered L-tryptophan in the body of nephrotic rats is still unclear. Therefore, we examined the changes in liver, kidney, brain, and muscle L-tryptophan levels after oral administration of L-tryptophan (100 μmol/kg) in rats with and without nephrosis, which was conducted 8 days after injection of puromycin aminonucleoside (100 mg/kg). Before L-tryptophan administration, liver and kidney L-tryptophan levels were higher in nephrotic rats than in non-nephrotic rats. At 15 min after L-tryptophan administration, there were no differences in increased amounts of kidney, brain, and muscle L-tryptophan and liver trypotphan 2,3-dioxygenase activity between rats with and without nephrosis, while an increased amount of liver L-tryptophan was much larger in nephrotic rats than in non-nephrotic rats. At 30 min after L-tryptophan administration, liver L-tryptophan level was similar in rats with and without nephrosis and liver tryptophan 2,3-dioxigenase activity was higher in nephrotic rats than in non-nephrotic rats, although an increased amount of L-tryptophan was similar in the kidney, brain and muscle of rats with and without nephrosis. These results indicate that elevated blood L-tryptophan after oral L-tryptophan administration to nephrotic rats is mainly transported to the liver and the transported L-tryptophan is actively metabolized in the tissue thereafter.

Correlations between serum concentrations of asymmetric (ADMA) and symmetric (SDMA) dimethylarginine (DMA) in patients with renal insufficiency and their concomitant diseases

Christian Fleck[1], **Frank Schweitzer**[1], **Elke Karge**[1], **Martin Busch**[2], and **Günter Stein**[2]

[1] Institut für Pharmakologie und Toxikologie,
[2] Department of Internal Medicine IV, Klinikum der Friedrich-Schiller-Universität, D-07740 Jena, Germany

NO synthesis is inhibited by ADMA, which accumulates, similar to SDMA, in the plasma of patients suffering from chronic renal failure. ADMA, and possibly SDMA, contribute to hypertension and atherosclerosis in renal patients: ADMA inhibits directly eNOS, whereas SDMA competes with the NO precursor arginine for uptake into the cells. In 26 controls and 221 patients with kidney diseases as were chronic renal failure (CRF), end stage renal disease, and patients after renal transplantation (RT), the plasma concentrations of ADMA, SDMA, and 20 endogenous amino acids were measured and correlated to blood pressure, cardiac diseases, diabetes mellitus, and endothelial dysfunction. Both ADMA (1.04 vs. 0.66 μM) and SDMA (2.69 vs. 0.49 μM) were significantly elevated in all patients compared to healthy controls whereas arginine concentration (51.4 vs. 76.0 μM) was decreased in dependence on the degree of kidney disease. In RT patients, SDMA levels significantly decreased, but ADMA remained enhanced. A strong correlation was found between SDMA and both serum urea and creatinine. There was no strong correlation between the underlying renal disease and DMAs concentrations. A linear correlation was found between ADMA and cholesterol concentrations in RT patients. Hypertension in CRF was accompanied by a further increase in the concentration of DMAs. There was no relation between DMAs and the occurrence of arterial occlusive disease, cerebrovascular insufficiency, and phlebothrombosis. In patients with cardiac diseases only SDMA was additionally increased in CRF. Correlations between ADMA or SDMA and cardiovascular or other concomitant diseases are of minor importance.

Substrates of the neutral amino acid transport system N interfere with the arginine supply of nitric oxide synthase in human endothelial cells

Alexandra Simon, Alice Habermeier, Lars Plies, and **Ellen I. Closs**

Department of Pharmacology, Johannes Gutenberg University, 55101 Mainz

Our previous studies in human endothelial EA.hy926 cells suggested the existence of two L-arginine pools: Pool I can be depleted by extracellular L-lysine, whereas pool II is not freely exchangeable with the extracellular space, but accessible to endothelial NOS (eNOS). We hypothesized that the L-arginine pool II can be explained by efficient recycling of L-arginine from L-citrulline since L-citrulline was able to enhance NO production. Here we studied the influence of cationic and neutral amino acids on the L-arginine pool II in endothelial cells. While the intracellular L-arginine concentration in EA.hy926 dropped to less than 1% after a 30 min incubation in 1 mM L-lysine, NO synthesis was only reduced to 40–60%. In contrast, after a 2 h incubation in L-lysine,

there was no decrease in NO production. The 30 min incubation in L-lysine did not result in a decrease in NO synthesis when cells had been preincubated in DMEM without L-glutamine. Also, L-glutamine present during L-lysine incubation enhanced the inhibitory effect. Based on these data we hypothesized the existence of a transport system that exchanges extracellular L-glutamine for intracellular L-citrulline. We found that only neutral amino acids that match the substrate profile of system N transporter 1 (SN1) were able to reduce NO synthesis and that SN1 was expressed in EA.hy926 cells. Similar results were obtained in HUVECs. In conclusion, we demonstrate that the inhibitory effect of L-glutamine on NO synthesis is based on the removal of intracellular L-citrulline, presumably via SN1.

Modulation of L-arginine transport by elevated D-glucose and adenosine in human fetal endothelium

Paola Casanello[1,2], **Felipe Sanhueza**[1,3], **Marcelo González**[1,3], and **Luis Sobrevia**[1]

[1] Cellular and Molecular Physiology Laboratory (CMPL), Department of Obstetrics and Gynecology and Medical Research Centre (CIM), School of Medicine, Faculty of Medicine, Pontificia Universidad Católica de Chile, P.O. Box 114-D, Santiago,
[2] Department of Obstetrics and Gynaecology, Faculty of Medicine, and
[3] Faculty of Pharmacy, University of Concepción, P.O. Box 160-C, Concepción, Chile

L-Arginine transport and nitric oxide synthesis are increased in human umbilical vein endothelial cells from pregnancies with gestational diabetes and in endothelial cells isolated from normal pregnancies exposed to 25 mM D-glucose. Gestational diabetes and elevated D-glucose are associated with reduced transport of the endogenous nucleoside adenosine in human fetal endothelium. This study describes cell signalling mechanisms associated with modulation of L-arginine transporters by D-glucose and adenosine in human endothelium. Human umbilical vein endothelial cells were cultured in medium 199 containing 20% newborn- and fetal calf serum. L-Arginine transport (1 min, 2 μCi/ml, 37°C) was determined in Krebs solution containing 5–40 mM D-glucose (0.5 min–24 h) or 0.1–100 μM adenosine. mRNA for system y^+/CATs (Cationic Amino acid Transporters) were quantitated by real time PCR. D-Glucose and adenosine increased the V_{max} for L-arginine transport, with no changes in the apparent K_m. D-Glucose also increased mRNA level for human CAT-1 and CAT-2B transporters, but reduced human equilibrative nucleoside transporters 1 (system *es* or hENT1) mRNA and protein levels, and activity. D-Glucose and adenosine effects involved activation of protein kinase C, the mitogen-activated protein kinases ERK1 and ERK2, and endothelial nitric oxide. A_{2a} purinoceptor antagonists blocked D-glucose and adenosine effects. These results suggest that elevated D-glucose-increased L-arginine transport activity may result from a reduced expression and activity of equilibrative nucleoside transporters leading to activation of purinoceptors in human fetal endothelial cells.

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Short term regulation of aminoisobutyric acid transport by thyroid hormones during prenatal life in hepatocytes

Silvana Spagnuolo[1], **Alessandra Spaziani**[2], and **Silvia Leoni**[2]

[1] Department of Pharmacology of Natural Substances and General Physiology,
[2] Department of Cellular and Developmental Biology, University of Rome ''La Sapienza'', 00185 Rome, Italy

Prenatal development is characterized by high amino acid requirement to support organ and body growth.

Actually hormonal control of amino acid uptake during development is little known. In this work we analysed the short term effect of thyroid hormones 3,5,3′-Triiodotyronine and L-Thyroxine and the analogue 3,5-Diiodo-thyronine on system A mediated amino acid transport (assayed as ^{14}C-Na^+ dependent Aminoisobutyric acid uptake) and the signal transduction pathways involved. We used cultured chick embryo hepatocytes at 14 and 19 days of prenatal life because these cells are in different phases of cell cycle and have different activities of deiiodinases.

Short term treatment of hepatocytes with 3,5,3′-Triiodotyronine, L-Thyroxine and 3,5-Diiodo-thyronine stimulates Aminoisobutyric acid uptake only at 19 days. This effect is blocked by Ro 31-8220, a Protein-Kinase-C inhibitor. An involvement of Protein-Kinase-C is suggested also by the increased levels of inositol-tri-phosphate and diacylglicerol detected after thyroid hormones treatment. Thyroid hormones increase Protein-Kinase-Cα and decrease Protein-Kinase-Cε activities indicating that the two isoforms differently modulate the transport. Incubation with PD98059, a Mitogen-Activated-Protein-Kinase inhibitor, decreases clearly the stimulatory effect of thyroid hormones, suggesting also a role of Mitogen-Activated-Protein-Kinase in the regulation of amino acid transport confirmed by the higher level of Mitogen-Activated-Protein-Kinase phosphorylation after thyroid hormone treatment.

Our results demonstrate that the hormonal regulation of system A is dependent on developmental stage and that thyroid hormones have non genomic effects on amino acid transport using Protein-Kinase-C and Mitogen-Activated-Protein-Kinase activation.

Moreover our data support the hypothesis that 3,5-Diiodo-thyronine can mimic the role of 3,5,3′-Triiodotyronine and L-Thyroxine as a new thyroid hormone.

hPAT1: a high capacity mechanism for taurine, betaine, imino acid and drug absorption across the human small intestine

David T. Thwaites[1] and **Vadivel Ganapathy**[2]

[1] School of Cell & Molecular Biosciences, The Medical School, Framlington Place, Newcastle upon Tyne NE2 4HH, UK
[2] Medical College of Georgia, Biochemistry & Molecular Biology, Augusta, GA 30912-2100, USA

Although oral absorption of taurine, betaine, imino acids and D-amino acids is known to occur across the human small intestine the identity and nature of the transport mechanism(s) has remained unclear. A transport system with similar substrate specificity to the rat imino acid carrier has been identified functionally at the apical membrane of the human intestinal epithelial cell line Caco-2. This transport mechanism was named ''system PAT'' (for Proton-coupled Amino-acid Transporter) as it functions in an H^+-coupled, pH-dependent, Na^+-independent manner. Recently a cDNA has been isolated from Caco-2 cells that is able to induce H^+-coupled amino acid transport (hPAT1, for human Proton-coupled Amino-acid Transporter 1). hPAT1 has an identical substrate specificity to that measured for system PAT in Caco-2 cell monolayers and the imino acid carrier in rat small intestine. Immunocytochemistry demonstrates PAT1-like immunoreactivity (IR) localised solely to the apical membrane of Caco-2 cells and human and rat small intestinal enterocytes. Substrates for this transporter include a range of small, unbranched, dipolar amino acids (including methylated analogues such as betaine and MeAIB), imino acids, beta amino acids (e.g. taurine), and potential neuromodulatory amino acids including D-amino acids (e.g. D-cycloserine and D-serine) and GABA (and analogues e.g. nipecotic acid). The presence of a high capacity transport system with such a broad range of transported substrates provides a route for nutrient, osmolyte and drug transport across the luminal brush-border membrane of the human small intestine. Supported by the MRC (grant G9801704) and BBSRC (grant 13/D17277).

Rat model of fatigue reduction and enhancement by controlling tryptophan level in the brain

T. Yamamoto[1] and **E. A. Newsholme**[2]

[1] Health Science Laboratory, Tezukayama University, Nara, Japan
[2] Merton College, Oxford, U.K.

A causing factor of central fatigue is related to the enhancement of serotonergic function. We report the effect on central fatigue when tryptophan, which is a precursor of serotonin, is increased. When 63 mg/Kg, tryptophan was injected into the rats peripherally, there was no significant change of central fatigue level. This was measured by comparing the exhaustion times of subjects while running on the treadmill to control groups. However, peripheral injection of 125 mg/Kg, tryptophan showed a significant decreased time to exhaustion. Thus, enhanced serotonergic function depended on the concentration of tryptophan given cause central fatigue. To certify the validity of this experiment as a measurement of central fatigue, we investigated exhaustion time after the injection of 125 mg/Kg of tryptophan. The effects of musical stimuli with this amount of tryptophan was studied at the same time. We thought that a musical stimulant fluctuated at $1/f$ may have an effect on the reduction of central fatigue. The results were that a $1/f$ fluctuation of musical stimuli with a tryptophan injection showed prolonged time to exhaustion, compared to a musical stimuli of white noise with the same amount of tryptophan also given by the same route. These results suggest that the combination of tryptophan loading and treadmill running with a musical stimulant is a useful animal model for the study of central fatigue.

Analysis

Determination of biogenic amines in tea

J. Kirschbaum, S. Flassig, and **H. Brückner**

Interdisciplinary Research Center, Department of Food Sciences, University of Giessen, Heinrich-Buff-Ring 26, 35392 Giessen, Germany

Samples of tea (*Camellia sinensis*) of various countries and regions of origin (India, Ceylon, China, Assam, Darjeeling) were analyzed for the presence of eight nonvolatile biogenic amines.

Quantities of amines were determined in boiling water extracts of tea (14 black, 5 green and 1 Oolong tea). Amines were derivatized with 9-fluorenylmethoxycabonyl chloride (FMOC-Cl). The resulting derivatives were resolved on a Supersphere 60 RP-8 (Merck) octasilyl column using a binary gradient generated from 0.1 M sodium acetate (pH 7.0) and acetonitrile and detected by their fluorescence. In the Oolong tea and one black tea no biogenic amines could be detected (detection limit 0.07–1.0 pmol for biogenic amines at signal-to-noise ratio 3), whereas in the other teas varying, albeit low, amounts of biogenic amines were found.

Quantities of biogenic amines ranged from totally 0–504 μg/L infusions prepared according to standard protocols ISO 3103 or BS 6008 (i.e. 2.8 g tea leaves in 140 mL boiling water, corresponding to 20 g tea leaves in 1 L water). Since no histamine was detected in tea infusions and total quantities of biogenic amines determined are much lower in comparison to other foods and beverages such as fish, seafood, cheese or wine, no health risks related to these compounds are to be expected on consumption of tea as beverage.

Amino acid analysis of Asian fermented foods using HPLC and precolumn derivatization with OPA/MPA/FMOC-Cl

J. Kirschbaum[1], **I.-G. Baek**[1], **W.-D. Beinert**[2], and **H. Brückner**[1]

[1] Interdisciplinary Research Center, Department of Food Sciences, University of Giessen, Heinrich-Buff-Ring 26, D-35392 Giessen, Germany
[2] Merck Comp., Darmstadt, Germany

For the production of Asian fermented foods and beverages indigenous microorganisms (bacteria, yeasts, molds) are used. Their action and metabolites are responsible for the final flavor and texture of foods including seasonings and condiments. Amino acids are resposible for nutritional value and taste and serve as precursors for biogenic amines.

The amino acid pattern can also be used for quality assessment and authenticity control of foods.

We demonstrate that free amino acids can be analysed by HPLC using fully automated precolumn derivatization with *o*-phthaldialdehyde (OPA) together with 3-mercaptopropionic acid (MPA). Since secondary amino acids (proline, hydroxyproline) do not react with OPA the reagent 9-fluorenylmethoxycarbonyl chloride (FMOC-Cl) was added to derivatizing reagents. Norvaline and sarcosine were used as internal standards. For HPLC a LaChrom instrument (Merck-Hitachi, Darmstadt, Tokyo) was used comprising an autoderivatizer, pump with low pressure gradient former, and column oven. For the detection and quantification of amino acid derivatives a programmable fluorescence detector was employed.

Derivatives were resolved on a Nov-PAK C18 column using an binary gradient generated from 0.1 M sodium acetate/0.044% triethylamin (pH 6.5) and acetonitrile. Data processing was performed using D-7000 HPLC System Manager and software. This method allowed in a single run the reliable and rugged separation and quantification of 23 food amino acids, including citrulline, ornithine, taurine and gaba, as well as the internal standards norvaline and sarcosine. The methods was applied to quantify amino acids in Asian fermented foods such as soy sauce, fish sauce, miso, and sake.

Design of reagents for amino acid analysis based on cyanuric chloride

H. Brückner and **M. Wachsmann**

Interdisciplinary Research Center, Department of Food Sciences, University of Giessen, Heinrich-Buff-Ring 26, 35392 Giessen, Germany

Owing to its trifunctionality, cyanuric chloride (1,3,5-trichloro-*s*-triazine, *s*-triazine) is a versatile starting compound for the design of reagents suitable in particular for amino acid analysis. We show that two chlorine in cyanuric chloride can be replaced successively by (i) Friedel-Crafts reaction with 1-methoxynaphthalene, and (ii) by reaction with methanol. The monofunctional, highly fluorescent reagent 2-chloro-4-methoxy-6-(4-methoxy-1-naphthyl)-[1,3,5]triazine is suitable for the fluorescence labeling of proteins or for the derivatization of amino acids followed by their liquid chromatographic separation. This approach was extended to the synthesis of a series of chiral derivatizing reagents (CDRs) suitable for the derivatization of amino acid enantiomers. In a first step one chlorine in cyanuric chloride was replaced by an alkoxy or aryloxy group such as methoxy, nitrophenoxy, or methylcoumaryloxy. Then a second chlorine was replaced by L-valine amide oder L-phenylalanine amide serving as chiral residues. The resulting CDRs having a remaining reactive chlorine were used for the derivatizing of mixtures of amino acid enantiomers followed by the liquid chromatographic separation of the resulting diastereomers. The approaches outlined make possible the design of chiral and nonchiral reagents utilizing an abundance

of UV-absorbing, chromogenic, fluorogenic, or otherwise suitable reporter groups.

Amperometric amino acid and protein bioprobe analysis in food and pharmaceutical products

L. Micheli, G. Volpe, D. Moscone, D. Compagnone, and **G. Palleschi**

Dipartimento di Scienze e Tecnologie Chimiche, Università di Roma ''Tor Vergata'', Via della Ricerca Scientifica, 00133 Roma, Italy

The study and the realisation of amperometric enzyme probes for L-lysine, glutamate and aspartate in food and pharmaceutical products, and for transaminases and alanine in serum are the aim of this work. The specific enzymes were immobilised on a preactivated support, which was placed on a working electrode and additional protective membranes were used to reduce the electrochemical interferences and to protect the immobilised enzymes. Measurements were carried out in batch and/or in flow systems.

For glutamate and aspartate analysis, both the enzyme L-glutamate oxidase and aspartate aminotransferase were covalently co-immobilised. The hydrogen peroxide produced in the reaction was oxidised at the platinum electrode and the current output was correlated to the concentration of glutamate or aspartate present in the sample. Since the same cell can be used for detection of the two metabolites, when both were present, glutamate was detected first without adding the alphaketoglutarate to the buffered solution. Then this co-substrate was added and the analysis of aspartate carried out.

For alanine analysis both aminotransferase and glutamate oxidase were immobilised on polymeric membrane supports. The results obtained for glutamate, aspartate and alanine analysis procedures are in good correlation with HPLC procedure.

For the analysis of transaminases in human serum, an amperometric glutamate biosensor was used. The probes was applied to the measurement of activities of aspartate and alanine aminotransferase in human serum. Transaminases activity measured in 80 sera correlated well with results obtained with a spectrophotometric procedure.

Advances in the *o*-phthalaldehyde derivatization of amino acids and amines applying various SH-group additives

Ibolya Molnár-Perl, R. Hanczkó, D. Kutlán, Y. Mengerink, A. Csámpai, F. Tóth, and **T. Törö**

Institute of Inorganic & Analytical Chemistry, L. Eötvös University, Budapest 112, P.O. Box 32, Hungary

In the last four years, special characteristics of the *o*-phthalaldehyde (OPA) amino acid (AA) and amine (A) derivatives were clarified in our laboratory applying as SH-group containing additives 3-mercaptopropionic acid (MPA), N-acetyl-L-cysteine (NAC), the most popular 2-mercaptoethanol (MCE) and finally also ethanethiol (ET). The first step in order to answer the believed to be lower stability of glycine, ß-alanine, GABA, histidine, ornithine and lysine was an exhaustive derivatization study. Changes in responses, have been followed primarily by HPLC using UV and fluorescence detections, simultaneously, as a function of the reaction time, performing in all cases, without exception, thoroughly the same derivatization conditions, making possible a realistic comparison in practical and theoretical terms equally. On the basis of responses it became clear that the first forming derivatives of these amino acids are transforming to further ones furnishing characteristic UV maximum values. The initially obtained derivatives furnish a UV maximum at 334 nm, while the maximum of the transformed one's are shifted to 339 nm. Calculating the total of responses of these more than one derivative providing AAs they manifest the same stability than all others. This phenomena pointed ahead to solve the mechanism of the formation of the multiple OPA-derivatives. Amines provided the same characteristics as the above-mentioned AAs. Stoichiometric and on-line HPLC/MS data confirmed that the reaction of OPA with the primary amino group containing compounds, having the $-CH_2-NH_2$ moiety, results in more than one OPA molecule containing derivative. Based on the reaction mechanism principle improved analytical conditions was suggested.

Amino acids as a basic source of flavours

F. Osman and **A. Farouk**

National Research Centre, Tahrir Street, Dokki, Cairo, Egypt

The Complex set of reactions known collectively as the Maillard reaction have traditionally been considered to be responsible for the generation of roasted, toasted or caramel-like aroma as well as for the development of browned surface in foods. The role of free amino acids in this reaction is well established whereas the part played by the bound amino acids still needs extensive studies. In addition, the utilization of low priced amino acids sources such as wheat germ (by-product in wheat milling operations) can be verified for the production of suitable aroma concentrates.

The present work is a trial to obtain pleasant cookies flavour concentrates via Maillard reaction of wheat germ as amino acid source and fructose. So, a mixture of ground wheat germ and fructose (7:3 by wt.) was heated for 30 minutes at 150°C in oil bath under efficient reflux conditions. The proper time and temperature that provide the highest intensity and stability of the desired aroma were adjusted by using a simple designed sniffing port. Thereafter, the volatile components of the desired mixture was isolated by simulation steam distillation/solvent extraction method. The obtained aroma concentrate was separated into neutral – acidic and basic fractions and subsequently analysed by GC/MS. The components separated were 212 out of which 152 component could be identified. The obtained results indicated that the basic fraction comprises different classes such as pyrazines, pyridines and oxazoles with two major pyrazines namely, 2-ethyl-5-methyl pyrazine (8.65%) and 2-ethyl pyrazine (2.20%), while 2-acetyl pyridine (3.25%) represents the major pyridines. In the neutral-acidic fraction, the identified components include furans, furanones, pyrans, pyrroles and sulfur components in various percentages. As for the total aroma concentrate, the GC/MS showed almost all varieties of components in variable percentages. Aroma panel evaluation test is carried out on ten experienced panialists. The results are represented in suitable tables and figures and are discussed in details.

Enthalpic heterogeneous pair interaction coefficients between the zwitterions of the L-α-amino acids and a molecule of urea in water as a hydrophobicity parameter of amino acid side chains

Bartlomiej Pałecz

Department of Physical Chemistry, University of Lodz, Poland

The side chains of amino acids in solution react in various ways with the water molecules which surround them as well as with other components of solution depending on whether they possess non-polar, polar or ionic groups. Many research laboratories carry out studies intend to describe precisely the intermolecular interactions with the participation of amino acid side chains.

Such a description may allow one to better describe the spatial structures of protein and the mechanisms of folding its surface area.

The present work reports the results of calorimetric measurements of the enthalpies of dissolution of L-α-amino acids (glycine, alanine, aminobutric acid, valine, leucine, isoleucine, phenylalanine, tyrosine, methionine, proline, serine, threonine, cysteine, histidine, asparagine, glutamine, arginine, glutamic acid and tryptophane) in water and aqueous solutions of urea at the temperature of 298.15 K. Based on the obtained results, standard solutions enthalipes of L-α-amino acids in the examined solutions were determined. Using modified McMillan-Mayer's theory, these values were used to find the enthalpic heterogeneous interaction coefficient h_{AU}, which characterises the mutual interaction between the amino acid zwitterion and urea with the competitive participation of water molecules. Thus, these coefficients illustrate the differences of amino acid molecules interactions with the urea molecule and water molecules around them, and consequently they may play the part of a parameter, which differentiates the hydrophobic/hydrophilic properties of amino acid side chains.

The enthalpic heterogeneous interaction coefficients h_{AU} were compared also with the homogeneous interaction pair coefficients of L-α-amino acids h_{AA} (J. Am. Chem. Soc., 6003, 124, 2002).

Comparison of serum and plasma amino acid concentrations in humans

H. T. Pitkänen[1,2], **S. S. Oja**[3,4], and **A. A. Mero**[1]

[1] Department of Biology of Physical Activity, University of Jyväskylä, Jyväskylä, Finland
[2] Kankaanpää Rehabilitation Centre, Kankaanpää, Finland
[3] University of Tampere Medical School, Tampere, Finland
[4] Department of Clinical Physiology, Tampere University Hospital, Tampere, Finland

The amino acid concentrations in blood serum and plasma in medically screened healthy males, 33.3 ± 3.8 (mean $\pm$ SD) years ($n = 12$), and females, 32.6 ± 4.4 years, ($n = 12$) were compared. Each subject fasted from 8:30–10:30 pm to 06:30–08:30 am (at least 10 hours) before the tests, and was instructed to eat according to their normal dietary habits and to avoid any nutritional supplements that could increase protein or energy intake. The samples (10 ml) were taken from the antecubital vein to dry (serum) or heparin tubes (plasma) and centrifuged for 10 min at 3500 rpm to spin down blood cells. The supernatants were immediately frozen and stored at -20°C until analysed within 2–4 weeks using Shimadzu (Japan) LC-10Avp liquid chromatograph. All assays were done in duplicate and the intra-assay variation was 1.6% to 2.9% for all amino acids. The total concentration of amino acids was significantly (14%; $P < 0.01$) higher in serum than in plasma. The concentrations of individual amino acids were also significantly (8–81%; $P < 0.05$–0.001) higher in serum than in plasma. When compared with women, the concentrations of total amino acids (9%; $P < 0.05$), cysteine (55%; $P < 0.05$), glutamine (16%; $P < 0.01$), leucine (12%; $P < 0.05$), phenylalanine (10%; $P < 0.001$) and tryptophan (16%; $P < 0.05$) were higher in men in both samples. The present findings indicate that the concentrations of amino acids are markedly higher in serum than in plasma samples, apparently mostly due to liberation of amino acids from platelets in serum samples.

Similarity of the cellular amino acid composition of a gene group coding 3,00–7,000 amino acid residues

Kenji Sorimachi[1] and **Teiji Okayasu**[2]

[1] Department of Microbiology and
[2] Center of Medical Informatics, Dokkyo University School of Medicine, Mibu, Tochigi 321-0293, Japan

We already showed that the basic pattern of cellular amino acid composition as conserved in various organisms from bacteria to mammalian cells is expressed by the ''Star shape'', and that, similarly, this basic pattern is obtained from the codon usage, assuming that all genes are expressed equally. However, the transcriptional yield of each gene in a cell must be different; how we might solve this problem has long puzzled to us.

In the present study, using a ''unit theory'' based on the similarity of each unit, we demonstrate that the amino acid compositions presumed from a codon usage of ''units'' consisting of 10–20 genes, or single genes coding more than 3,000 amino acid residues resemble that presumed from the all genes consisting of the whole genome in bacteria. The same result was obtained from 10,465 mouse genes based on the cDNA analysis. Further, the existence of a ''unit'' coding 3,000–7,000 amino acid residues has been proved mathematically by applying simulation analysis based on a random choice of amino acid from an amino acid pool. This apparent ''unit'' shows the characteristic pattern of amino acid composition expressed by the ''Star shape''. Thus, a gene group consisting of the whole genome is apparently constructed from similar repeating small ''units'' without boundaries, and these ''units'' are distributed equally throughout the genome. These ''units'', important in organisms present today, might also have been so in primitive life forms not present today. In addition, gene formation might be based on a random choice of amino acids.

D-Amino Acids and Racemization

Alanine racemase and D-amino acid oxidase in aquatic animals

Hiroki Abe

Department of Aquatic Bioscience, The University of Tokyo, Bunkyo, Tokyo 113-8657, Japan

Free D-alanine exists in large amount (3–50 μmol/g wet wt.) in the tissues of several invertebrates such as crustaceans and bivalve mollusks. Under high salinity stress, these animals largely accumulate D- and L-alanine irrespective of species, suggesting that they are major compatible osmolytes responsible for the intracellular isosmotic regulation.

A highly specific alanine racemase has been proven to catalyze the inter-conversion of D- and L-alanine in these animals and purified from two crustacean species and a mollusk. A cDNA encoding the enzyme was isolated from the muscle and hepatopancreas of kuruma prawn *Penaeus japonicus* for the first time in animal kingdom. The cDNA encoded 421 amino acid residues. The deduced amino acid sequence including catalytic tyrosine and lysine residues showed 27% identity with that of bacteria and yeast. The enzyme activity largely increased during molting of *P. japonicus*.

Fish species contain only a trace amount of D-amino acids in their tissues. D-Amino acid and D-aspartate oxidases were detected in the liver, kidney, and intestine of various fishes. For the former enzyme, which was inducible with the oral administration of D-alanine, a cDNA was also cloned and sequenced from the hepatopancreas of common carp *Cyprinus carpio*. The cDNA consisted of 1,294 bp including an open

reading frame of 1,041 bp that encoded 347 amino acids. The amino acid identity of the sequence was about 60% with that of mammals and 29% with that of bacteria. The enzyme expressed in *E. coli* showed extremely higher activity than that in carp tissues.

D-amino acid-containing bioactive peptides in amphibian skin

Donatella Barra, Giuseppina Mignogna, Rossella Miele, M. Luisa Mangoni, and **Maurizio Simmaco**

Istituto Pasteur-Fondazione Cenci Bolognetti, Ospedale S. Andrea, Dipartimento di Scienze Biochimiche, Università La Sapienza, Roma, Italy

The occurrence of a D-amino acid in peptides from a vertebrate species was accepted with some difficulty by the scientific community at the time when dermorphin was first isolated in 1981 by Vittorio Erspamer from *Phyllomedusa sauvagei* skin. The initial skepticism and the difficulty in extending the first observation originated in part from inadequacy of analytical methods. We set up a procedure for the separation of D- and L-amino acids by reverse phase HPLC, involving pre-column derivatization with a chiral reagent, and incorporated this method in a protocol for the determination of the chirality of amino acid residues in the course of subtractive Edman degradation of peptides. A number of different opioid peptides from several *Phyllomedusa* species were then described, with distinct affinities for μ- or δ-opioid receptors. All these amphibian peptides share the N-terminal sequence Tyr-Xaa-Phe, where Xaa is always a D-amino acid. The cDNA encoding for dermorphin presents a normal codon at the position where the D-amino acid is found in the end-product. Therefore, a post-translational reaction is expected to occur, whereby the configuration at the α-carbon atom is changed.

Later, antimicrobial peptides, the H-type bombinins, were isolated from skin secretions of different *Bombina* species that contained a D-amino acid in the second position of their sequences. Also in this case, cDNAs or genes presented a codon for the corresponding L-amino acid.

The main difference between these two classes of amphibian peptides is that, in the case of opioid peptides, only the D-amino acid containing peptide exists in nature and the synthetic all-L-amino acid-containing analogs are completely devoid of biological activity, whereas for bombinins H both isomers were isolated from skin secretions and found to have different activities. Thus, in the latter case, these post-translational modifications increase the array of defense weapon that protect amphibian species against microorganisms and predators.

Studies are being performed to identify the catalyst that favors this modification, to clarify its mechanism of action.

Racemization of amino acids in food as result of the Maillard reaction

H. Brueckner and **R. Paetzold**

Interdisciplinary Research Center, Department of Food Sciences, University of Giessen, Heinrich-Buff-Ring 26, D-35392 Giessen, Germany

We had shown that heating of protein L-amino acids (2.5 mM in 5% aq. AcOH, pH 2.5) together with an excess of glucose or fructose (278 mM) at 100°C for 24–96 h lead to a much higher racemization in comparison to a standard in which saccharides were omitted (Amino Acids 2001, 21:429). The reaction of reducing sugars with amino components such as amino acids is known as the Maillard reaction or non-enzymatic browning of foods. At the very beginning of the reaction labile Schiff bases are formed. The reaction proceeds with the formation of *N*-glycosyl amino acids which rearrange to relatively stable Amadori and Heyns compounds (fructose amino acids). Fructose amino acids are common in certain foods and are already formed under mild conditions. The reaction proceeds with the formation of characteristic flavor compounds and melanoidins. It is postulated that in Schiff bases, rearrangement products, and in the course of the formation of deoxyosones azomethin carbanions are formed reversibly via proton abstraction. Reattachment of protons is accompanied by racemization of bonded amino acids. In the course of the reaction mixtures of L- and D- amino acids are released. At the final stages of the reaction amino acids are more or less exhausted. Presence of large amounts of reducing sugars, elevated temperature and low water activity favours racemization of amino acids. The Maillard reaction explains the occurrence of D-amino acids in food and drinks such as dried fruits, concentrated plant juices and saps, and fortified wines.

Gas chromatographic determination of D-amino acids in fortified wines

R. Pätzold, A. Nieto-Rodriguez, and **H. Brückner**

Interdisciplinary Research Center, Department of Food Sciences, University of Giessen, Heinrich-Buff-Ring 26, D-35392 Giessen, Germany

Quantities of free D-amino acids were determined in fortified (dessert) wines ($n = 41$) such as Port, Sherry, Malaga, Moscatel, Madeira, and Marsala. D-amino acids were isolated by treatment with cation exchanger and converted into volatile *N(O)*-pentafluoropropionyl amino acid 1-propyl esters for analysis. Enantiomers were separated by capillary gas chromatography on Chirasil-L-Val capillary columns and monitored by flame-ionization detection or selected-ion monitoring mass spectrometry. Major amounts of D-Ala (4.0–58.1%), D-Asp (8.9–51.3%), D-Glu (4.9–28.4%), and D-Ser (0.4–14.7%) were detected in all wines and minor amounts ($< 1\%$) of D-Lys, D-Phe, D-Tyr, and D-Val in some wines. Notably, relative quantitites of D-Ala exceeded 50% in some fortified wines. Much lower amounts of D-amino acids were detected in table wines. Despite the varying enological procedures typically for the production of fortified wines is the use of grape must rich in glucose and fructose, arresting of fermentation by addition of brandy, addition of concentrated grape must, and maturation of wines at elevated temperature. These conditions favor the Maillard reaction, i.e. the reaction of reducing sugars with amino acids. Consequently, this reaction is considered a major source for the generation of D-amino acids in dessert wines and related drinks.

Sensitive one-step HPLC method for determination and quantification of D-Asp and N-methyl-D-Asp

George Fisher, Mara Tsesarskaia, and **Erika Galindo**

Department of Chemistry, Barry University, Miami Shores, FL 33161, USA

Gyula Szókán, Institute for Organic Chemistry, Eotvos University, Budapest, Hungary

The unusual amino acids D-aspartate (D-Asp) and N-methyl-D-aspartate (NMDA) have been found in the neuroendocrine systems of vertebrates and invertebrates where they are believed to have a role in hormone release and synthesis, and memory and learning processes such as those associated with neurological disorders, for example: Alzheimer's disease. A number of different methods have been used to detect these amino acids. However, in order to detect NMDA, these methods require pretreatment of the sample with *o*-phthaldialdehyde (OPA) to remove primary amino acids which interfere with the detection of NMDA. Therefore, further advances in the study of D-Asp and NMDA require development of more reliable and sensitive methods for their determination and quantification. We report here a one step derivatization procedure with the chiral reagent N-α-(2,4-dinitro-5-fluorophenyl)-(D or L)-

valine amide (FDNP-Val-NH_2, a close analog of Marfey's reagent but with much higher molar absorptivity). The diastereomers formed are separated on an inexpensive reversed phase ODS-Hypersil column with elution by 0.1% TFA/water C 0.1% TFA/MeCN (90:10) for 2 min isocratically, followed by a linear gradient up to 70% TFA/MeCN. UV absorption at 340 nm permits detection levels in the range of 50–100 picomoles. The NMDA peak is not obscured by those of primary and other secondary amino acids (or even GABA and taurine); hence the removal of other amino acids by pretreatment with OPA is not required. This method is highly reliable and fast (overall 50 minutes run). Using this method, we have detected NMDA in several biological tissues (for example: brain, optical lobe, and bucchal mass of octopus), confirming the results of other researchers.

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An improved HPLC method for determination of D-aspartic acid

Erika Galindo, Kevin Peterson, Tiffany Goff, Mara Tsesarskaia, and **George Fisher**

Department of Chemistry, Barry University, Miami Shores, FL 33161, USA

D-Aspartic acid (D-Asp) is an endogenous amino acid that occurs in the neuroendocrine system of mammals where it is involved in hormone release and synthesis, and learning and memory processes associated with neurological disorders such as Alzheimer's disease. Several methods have been reported for determination of D- and L-Asp in amino acid mixtures by high performance liquid chromatography (HPLC) after derivatization with chiral fluorogens, e.g. adducts of D/L-Asp with *o*-phthaldialdehyde (OPA) and *N*-acetyl-L-cysteine (NAC) to form diastereomers which are separated on a reversed phase C-18 column using isocratic or gradient elution with sodium acetate-methanol (NaOAc–MeOH), and fluorescence detection. We have found that sodium citrate-methanol (NaCit–MeOH) gives better resolution in a shorter time than NaOAc–MeOH. We investigated various concentrations of NaCit from 20 to 50 mM, pHs from 5.0 to 6.5, various percents of MeOH from 5 to 15%, and flow rates from 0.75 to 1.5 mL/min. Optimum resolution of the diastereomers on a 25×0.46 mm ODS-Hypersil column was found using 30 mM NaCit, pH 5.5, 10% MeOH, at 1.0 mL/min. Using these conditions, 5 picomoles of D-Asp are well separated, by 1.3 minutess, from 100 picomoles of L-Asp (D-Asp elutes at 6.1 min and L-Asp at 7.4 min). Fluorescence responses (peak areas) of both the D- and L-Asp derivatives are linear over the range of 5 to 1000 picomoles per injection, and the specific fluorescence of the D-Asp is always about $12 \pm 1\%$ lower than that of L-Asp. This method is suitable for determination of small concentrations of D-Asp in biological tissues.

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Spontaneous D-amino acid formation in elderly human tissues

Noriko Fujii[1], **Katsunori Kuge**[1], **Shingo Tajima**[2], and **Tadashi ShimoOka**[3]

[1] Research Reactor Institute, Kyoto University,
[2] Department of Dermatology, National Defense Medical College, and
[3] Life Science Center, Asahi Techno Glass Corp.,
Funabashi 273-0044, Japan

Proteins have been considered to consist exclusively of L-amino acids in living tissues. However, we have previously shown Asp-151 and Asp-58 residues invert to the D-isomers at a high degree (D/L > 1.0) in alpha A-crystallin from the human eye lens during aging. The reaction was also accompanied by isomerization to the beta-Asp (isoaspartate) residues. Similar reactions were also reported in beta-amyloid protein of Alzheimer's disease. In the present study, we prepared a polyclonal antibody against D-beta-Asp containing peptide and examined its immunoreactivity in the skin. The antibody recognized integrated or disintegrated elastic fibers in the sun-exposed skin but not in the sun-protected skin of the elderly donors. Western blot analysis of the proteins isolated from sun-damaged skin demonstrated that the 50 kDa protein was immunoreactive with both antibodies for D-beta-Asp containing peptide and elastin. These results suggested that D-beta-Asp containing protein in skin may be elastin. Therefore, we synthesized three Asp-containing model peptides corresponding to sections of elastin: GVDAAAA, REGDPSSS, AGADEGVR and then analyzed the kinetics of Asp racemization in these peptides. We next investigated a possible mechanism involving irradiation. Ultraviolet (UV) irradiation on normal skin caused the appearance of D-beta-Asp containing peptide-immunoreactive fibers in the dermis. These results suggest that UV irradiation is closely related to the formation of D-beta-Asp in the elastic fibers of skin. We propose that D-beta-Asp could be a useful indicator not only for aging but also for sun damage of the skin.

Serum levels of D- and L-serine in the patients with schizophrenia

Takeshi Fukushima[1], **Kenji Hashimoto**[2], **Masaomi Iyo**[2], **Toshimasa Toyo'oka**[1], and **Kazuhiro Imai**[3]

[1] Department of Analytical Chemistry, School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada Shizuoka 422-8526, Japan
[2] Department of Psychiatry, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chiba 260-8670, Japan
[3] Center for Research and Development, Kyoritsu College of Pharmacy, 1-5-30 Shibakoen, Minato-ku, Tokyo 105-8512, Japan

Several lines of evidence suggest that D-serine, which is synthesized from L-serine by the serine racemase in human, may function as an endogenous agonist for glycine binding site in NMDA receptor. The hypofunction of NMDA subtype receptors has been implicated in the pathophysiology of schizophrenia. In this study, we determined serum D- and L-serine simultaneously in the patients with schizophrenia by means of pre-column fluorescence derivatization and column-switching HPLC system consisting of ODS and chiral columns. As a result, the serum levels of D-serine in the patients with schizophrenia were significantly decreased as compared with those in the age- and gender-matched normal controls ($p = 0.001$). In contrast, the serum levels of L-serine in the patients were significantly increased as compared with those in the controls ($p = 0.013$). In addition, the percentage of D-serine in total (D + L)-serine in the patients was significantly decreased as compared with that in the controls ($p < 0.0001$), suggesting that the activity of serine racemase may be reduced in the patients. The present data suggest that decreased amount of D-serine may play a role in the pathophysiology of schizophrenia, and that serum levels of D- and L-serine would be a biological marker for schizophrenia.

A new chiral thiol reagent for automated precolumn derivatization and HPLC enantioseparation of amino acids. Application to the aspartate racemase assay

H. Homma[1], **N. Nimura**[1], **T. Fujiwara**[1], **A. Watanabe**[1], **M. Sekine**[1], **T. Furuchi**[1], **M. Yohda**[2], **A. Yamagishi**[3], and **T. Oshima**[3]

[1] School of Pharmaceutical Sciences, Kitasato University,
[2] Tokyo University of Agriculture and Technology, and
[3] Tokyo University of Pharmacy and Life Sciences, Japan

A novel optically active thiol compound, N-(*tert*-butylthiocarbamoyl)-L -cysteine ethyl ester (BTCC), is synthesized as a chiral derivatization

reagent. With this compound and *o*-phthalaldehyde (OPA), amino acid enantiomers are derivatized to fluorescent diastereomers that are readily separable on a reverse phase column by HPLC. Enantioseparation of acidic amino acids in particular is markedly improved using BTCC. The HPLC method for enantioseparation with the novel compound is applied to the aspartate (Asp) racemase assay. Derivatized D-Asp is eluted before the L-Asp derivative, therefore a small amount of D-Asp produced by the activity of racemase on a large quantity of L-Asp substrate may be quantified accurately, even at very low activity. Since the derivatization reaction proceeds rapidly at room temperature, a fully automated system is established for the derivatization and sample injection. The automated method is practical and successfully applied to the assay of *Thermoplasma acidophilum* Asp racemase. The procedure is presumably applicable to the enantioseparation of other amino acids, amino alcohols and catecholamines.

Mouse serine racemase: gene structure and expression

Ryuichi Konno[1], **Nobuyoshi Akimitsu**[2], **Kenji Hamase**[3], and **Kazuhisa Sekimizu**[2]

[1] Department of Microbiology, Dokkyo University School of Medicine,
[2] Graduate School of Pharmaceutical Sciences, University of Tokyo, and
[3] Graduate School of Pharmaceutical Sciences, Kyushu University, Japan

Until recently D-amino acids have been considered not to be present in higher animals. However, it becomes evident that a large amount of D-serine is present in the brains of mammals. D-Serine binds to the strychnine-insensitive site of NMDA subtype of glutamate receptors and potentiates NMDA receptor activity. It functions as a modulator of neurotransmission. It is produced from L-serine by serine racemase. However, it is not known much about this enzyme. Therefore, we have examined the gene structure and expression of mouse serine racemase.

Mouse serine racemase cDNA was amplified using RT-PCR. Using this cDNA, Southern hybridization was carried out. Restriction fragment length polymorphism was not detected in seven strains of mice, indicating that these mice had the serine racemase gene of the similar structure. Lambda genomic library was screened for the serine racemase gene and three positive clones were isolated. Analysis of these clones indicated that serine racemase gene spanned approximately 17 kb and consisted of 9 exons. FISH mapping localized the serine racemase gene to 11qB4-5. Northern hybridization indicated this gene was expressed in many organs. High level of expression was observed in the liver and kidney, moderate level of expression in the heart, brain and testis, and low level of expression in the spleen, lung and skeletal muscle.

Storage of peptides in the skin of *Phyllomedusa bicolor*

C. Auvynet[1], **N. Seddiki**[2], **M. Amiche**[1], **P. Nicolas**[1], and **C. Lacombe**[1]

[1] Laboratoire de Bioactivation des Peptides, Institut Jacques Monod, CNRS, Université Paris 6-Paris 7, 2 Place Jussieu, 75251 Paris Cedex 05, France
[2] Laboratoire d'Ingénierie des Anticorps, Institut Pasteur, 28, rue du Docteur Roux, 75015 Paris, France

The skin of South American frogs contains large amounts of a wide range of biologically active peptides that are either identical or highly homologous to hormones or neurotransmitters of higher vertebrates. We have described several intriguing peptides from the skin of arboreal frogs Phyllomedusa bicolor and sauvagii. Most of them are derived from a group of peptide precursors collectively known as the preprodermorphin/dermaseptins. These D-amino acid-containing heptapeptides are very potent and exquisitely specific agonists of the μ- or the δ-opioid receptors. In contrast, the dermaseptin B and S families are α-helical amphipathic peptides that are broad-spectrum microbicides, but have little or no harmful effect on differentiated mammalian cells. The morphological and functional complexity of amphibian skin has been assessed in several studies but it is not clear whether or not the diverse pharmacologically active substances produced by amphibian skin are all secreted by the same type of gland. Moreover, if the two types of peptides (opioid and antimicrobial peptides) are elaborated in the same type of glands, it would be of greatest interest to know if both are packed in the same granules. Lastly how such identical precursors can lead to so different maturation processes, one resulting in D-amino acid containing peptides whereas the other yielding to α-helical amphipatic peptides? Using a specific antibody to the common preproregion of preprodermaseptins and preprodeltorphins, we already were able to locate these precursors in a specific type of serous gland during development as well. We used polyclonal antibodies against dermaseptin, and monoclonal antibodies against deltorphin to establish if the same serous gland is able to produce both peptides, and to detect if they are synthetized and stocked in the same type of granules.

Glutathione and Glutathione-S-Transferases in Toxicology

Specific interaction of a natural NO carrier with the glutathione transferase superfamily

Francesca De Maria[1], **Paola Turella**[1], **Anna Maria Caccuri**[1], **Giovanni Antonini**[2], **Lorenzo Stella**[1], **Giorgio Federici**[3], and **Giorgio Ricci**[1]

[1] Department of Chemical Sciences and Technologies, University of Rome "Tor Vergata", Italy
[2] Department of Biology, University of Rome "Roma Tre", Italy
[3] Children's Hospital IRCCS "Bambin Gesù", Rome, Italy

The interaction of dinitrosyl-diglutathionyl iron complex (DNDGIC), a natural carrier of nitric oxide, with representative members of the human glutathione transferase superfamily, i.e. GSTA1-1, GSTM2-2, GSTP1-1, GSTT2-2 and GSTB1-1 has been investigated. GSTA1-1 shows the strongest interaction ($K_D = 10^{-10}$) while GSTM2-2 and GSTP1-1 both have ten times lower affinity. Pre-steady state and steady state binding data fit well to a minimal mechanism with one high and one low affinity binding site. Binding of the complex to the first subunit of GST triggers structural intersubunit communication which lowers the affinity for DNDGIC in the vacant subunit. The negative cooperativity does not affect the catalytic competence of the vacant subunit in GSTP1-1 and GSTM2-2 but causes a drastic loss of activity in GSTA1-1. Interestingly, the ancestral Theta class GSTT2-2 shows a very low affinity for the complex ($K_D = 10^{-7}$), whereas, the bacterial GSTB1-1 is not inhibited by DNDGIC. The optimised interaction with this NO carrier, developed in the more recently evolved GSTs may be related to the acquired capacity of evolute organism to utilise NO as a signal trasduction messenger.

GSH depletion upregulate BCL-2 in BSO-resistant cells: possible involvement of NF-kappaB-mediated survival pathways

Maria D'Alessio[1], **Claudia Cerella**[1], **Milena De Nicola**[1], **Carla Amici**[1], **Caterina Pesce**[3], **Antonio Bermgamaschi**[2], **Giampiero Gualandi**[4], and **Lina Ghibelli**[1]

[1] Department of Biology,
[2] Department of Occupational Health, and
[3] Department of Experimental Medicine, University of Rome ''Tor Vergata'', via Ricerca Scientifica 00133 Rome, Italy
[4] DABAC, University of Tuscia, Viterbo, Italy

Apoptosis is the process through which anticancer drugs exert their citotoxic effect. The molecular mechanism underlying intrinsic drug resistance of some human tumors implies reduced apoptosis and may involve enhanced expression of antiapoptotic protein of the bcl-2 family and of cytoprotective, drug-detoxifying enzymes, including the glutathione system.

In this study, we investigate the involvement of Bcl-2 in the fate of cells deprived of glutathione (GSH) by the glutathione synthesis inhibitor buthionine sulfoximine (BSO). We have observed that in monocytic U937 cells, and hepatocytic HepG2 cells, BSO treatment upregulates Bcl-2 protein, in a protein synthesis dependent fashion. This is accompanied by increased levels of Bcl-2 mRNA. At the same time, BSO activates NF-kappaB. Inhibition of NF-kappaB strongly reduced the BSO-dependent Bcl-2 up-regulation, suggesting that Bcl-2 is transcriptionally transactivated in an NF-kappaB-mediated fashion.

In these cells BSO is not apoptogenic by itself. However, in the presence of specific NF-kappaB inhibitors, BSO becomes apoptogenic, indicating that cell survival to BSO is mediated by NF-kappaB.

In other cells, such as lymphoma cells Bl41, the mere BSO treatment is sufficient to induce apoptosis. Interestingly, in these cells BSO does not elicit Bcl-2 up-regulation.

Our study delineates for the first time an adaptive, NF-kappaB-mediated, upregulation of Bcl-2 in response to redox alteration, possibly linking survival to BSO to the ability to overexpress Bcl-2. Our model may help to delineate the mechanism involved in the most diffuse type of chemoreistance. Indeed, increased levels of Bcl-2 characterise most chemoresistent tumors; complementary, BSO is often used as chemosensitizer.

Different fates of intracellular glutathione determine different apoptotic morphologies

Milena De Nicola[1], **Giampiero Gualandi**[4], **Alberto Alfonsi**[4], **Claudia Fanelli**[1], **Claudia Cerella**[1], **Maria D'Alessio**[1], **Laura Teodori**[3], **Antonio Bergamaschi**[2], and **Lina Ghibelli**[1]

[1] Dipartimento di Biologia,
[2] Cattedra di Medicina del Lavoro, Universita' di Roma Tor Vergata, via Ricerca Scientifica 00133 Roma,
[3] Dipartimento Biomedicina e Tossicologia, ENEA, Roma, and
[4] DABAC, Universita' della Tuscia, Viterbo, Italy

The role of GSH efflux is particularly important in toxicology and oncology, because this is a key phenomenon in the resistance to cancer therapies as well as in the sensitivities to cytotoxic agents. We showed that the extrusion of GSH is a sine qua non for stress-induced apoptosis (i.e., with respect to physiological apoptosis). Here, we investigate whether different apoptotic routes/morphologies reflect the different redox requirements of the biochemical pathways of apoptosis. Indeed, U937 monocytic cells show two different apoptotic nuclear morphologies, budding and cleavage, that are the result of two independent morphological routes, since they never interconvert one into the other, and are differently modulated by stressing or physiological apoptogenic agents. *In situ* analysis showed that in U937 cells intracellular GSH is lost in cells undergoing cleavage, whereas it is maintained in apoptotic budding cells. Lymphoma cells BL41 lose GSH in apoptosis, and show the cleavage nuclear morphology; the same cells latently infected with Epstein Barr Virus (E2r line) undergo apoptosis without GSH depletion and show the budding nuclear morphology. In order to explore the sensibilization to chemotherapic treatments induced by agents that modulate the GSH efflux we show that GSH depletion is not only concomitant to, but is the determinant of, the cleavage morphology, since the inhibition of apoptotic GSH efflux with cystathionine increases the budding-to-cleavage ratio.

Induction of apoptosis by curcumin: mediation by glutathione S-transferase P1-1 inhibition

Marc Diederich

Laboratoire de Recherche sur le Cancer et les Maladies du Sang (RCMS), Centre Universitaire de Luxembourg, 162A, avenue de la Faïencerie, L-1511 Luxembourg, Luxembourg

Expression of glutathione S-transferase P1-1 (GSTP1-1) is correlated to carcinogenesis and resistance of cancer cells against chemotherapeutic agents. Curcumin, a natural compound extracted from Curcuma longa, has shown strong antioxidant and anticancer properties and also the ability to regulate a wide variety of genes that require Activating Protein 1 (AP-1) and Nuclear Factor kB (NF-kB) activation. In the present study, we examined the inhibitory effect of curcumin on the expression of GSTP1-1 mRNA as well as protein and we correlated this inhibition with the apoptotic effect of curcumin on K562 leukemia cells. Curcumin efficiently inhibited the Tumor Necrosis Factor a (TNFa)- and phorbol ester-induced binding of AP-1 and NF-kB transcription factors to sites located on the GSTP1-1 gene promoter. TNFa-induced GSTP1-1 promoter activity was also inhibited by curcumin as shown by reporter gene assay. In parallel, curcumin induced pro-caspases 8 and 9 as well as Poly ADP Ribose Polymerase (PARP) cleavage and thus leading to apoptosis in K562 cells. Our results overall add a novel role for curcumin as this chemoprotective compound could contribute to induce apoptosis by its ability to inhibit the GSTP1-1 expression at the level of transcription.

Glutathione in apoptotic and survival pathways

M. D'Alessio[1], **M. De Nicola**[1], **C. Cerella**[1], **A. Bergamaschi**[2], and **Lina Ghibelli**[1]

[1] Dipartimento di Biologia and
[2] Cattedra Medicina del Lavoro, Universita' di Roma Tor Vergata, Rome, Italy

A huge number of reports in the scientific literature connect glutathione and apoptosis, making the analysis of the roles glutathione plays in apoptosis quite complex. Glutathione interferes with apoptosis either in its detoxifying or antioxidant role, since the apoptotic process involve both redox alterations and the disposal/development of toxic compounds/metabolites.

As far as redox alterations are concerned, glutathione may detrmine the fate of cells induced to apoptosis playing multiple and sometimes contrasting roles.

For apoptogenic agents that directly induce oxidative stress, glutathione may scavange peroxides/radicals, and/or help in buffering the redox equilibrium altered by the stress, thus decreasing the extent of the insult and reduce apoptosis. In this instances, the intracellular GSH concentration regulates the trigger of apoptotic signaling.

In instances of apoptosis induced by non-oxidative agents, GSH may be part of the progression, rather than the triggering, of the apoptotic signaling, being actively extruded by specific carriers in the reduced

state, leading to oxidation of specific target proteins such as Bax and/or caspases. GSH efflux only modulate apoptosis in cells that possess and regulate the specific carriers.

Another point deals with the common practice of using GSH depleting agents as sensitizers for chemoresistant tumor cells. This is not always a useful therapeutical approach, since some cell types are able to survive slow, pharmacologically induced, GSH depletion. These GSH depleted cells are indeed able to adapt to the mild oxidative environment by overexpressing antiapoptotic proteins such as Bcl-2 or IAPs.

Glutathione as "sensor" of the apoptosis induced by DHA

Nicolò Merendino[1], **Gloria Pessina**[1], **Barbara Loppi**[1], **Romina Molinari**[1], **Massimo D'Aquino**[2], **Francesca Velotti**[1], and **Gianni Tomassi**[1]

[1] Department of Environmental Sciences, Tuscia University, 01100 Viterbo, Italy

[2] National Institute for Food and Nutrition Research (INRAN), 00178 Rome, Italy

The tripeptide glutathione (GSH) is the predominant low molecular weight thiol reductant in mammalian cells. GSH provides cells with their reducing milieu and protects them against the oxidative effects of ROS endogenously produced in cells by decaying H_2O_2 and lipid hydroperoxides. Moreover it seems to play an important role in the apoptosis induced by different stimuli, but the mechanisms of action are not completely clarified. In this study we investigate the mechanisms involved in apoptosis induced by Docosahexaenoic acid (DHA) (the fish oil rich fatty acid), utilizing as experimental model different human epithelial cell lines coming from pancreas, colon and urinary bladder.

Our results indicate that an early event in the apoptosis induced by DHA is the GSH extrusion without affecting oxidized glutathione (GSSG). Increased membrane lipid peroxidation seems to be implicated since vitamin E, BHT and vitamin C inhibit apoptosis induced by DHA.

Furthermore, lipoxygenase gene (LOX) modulation and its products (lipid hydroperoxides) play important roles in DHA induction of cell death caused by GSH depletion since LOX 15 and LOX-3 mRNA expression increases 6 hours after DHA treatment. Finally the agonists of the retinoid receptor RXR, 9-cis-retinoic acid and 13-cis retinoic acids are able to enhance the ability of DHA to induce apoptosis.

The results indicate the involvement of lipid peroxidation and of LOX gene modulation, probably due to the nuclear receptor PPARs and RXR activation, during DHA induced apoptosis through active GSH extrusion.

The regulation of tumor cell apoptosis by glutathione

Raymond E. Meyn and **Anupama Munshi**

Gus Pappas Department of Experimental Radiation Oncology, The University of Texas, M.D. Anderson Cancer Center 1515, Holcombe Boulevard-Unit 66, Houston, TX 77030, USA

Cell death by apoptosis is now understood to be a major cellular response to most modalities of cancer treatment including immunotherapy, chemotherapy, and radiotherapy. However, tumor cells become resistant to these treatments due to abnormal expression of certain oncogenes and tumor suppressor genes. The oncogene, Bcl-2, is the prototypic member of a family of proteins that regulate apoptosis. Bcl-2 expression by tumor cells results in an inhibition of their apoptotic response to therapeutics but the biochemical basis for this resistance remains obscure. We and others have observed that Bcl-2 expressing cells have higher intracellular levels of glutathione and that lowering these levels by a variety of means sensitizes the cells to apoptosis-inducing treatments. Such findings generally suggest that Bcl-2 may regulate apoptosis sensitivity by modulating glutathione metabolism. In this regard, we have been investigating the mechanism by which Bcl-2 affects cellular glutathione levels. In the past, we reported that Bcl-2's localization in the nuclear membrane resulted in higher glutathione levels in the nucleus thereby suppressing DNA fragmentation. Recently, we have extended our investigations to the mitochondria where Bcl-2's localization and may control the initiation of apoptosis. Our studies demonstrate that mitochondria isolated from Bcl-2 expressing cells have twice the level of glutathione as do control mitochondria. Further experiments have suggested that the presence of Bcl-2 in the mitochondrial membrane may regulate the transport of glutathione from the cytosol into the mitochondira. Once inside, glutathione may suppress the oxidative stress that leads to the release of apoptogenic factors.

Membrane γ-glutamyltransferase: from sulfur amino acid metabolism to cell redox regulation

S. Dominici, L. Pieri, M. Franzini, A. Paolicchi, and **A. Pompella**

Department of Experimental Pathology B.M.I.E., University of Pisa, Italy

γ-Glutamyltransferase (GGT) is the sole enzyme capable of cleaving the γ-glutamyl bond present in glutathione (GSH). The first step is thus provided in the degradation of extracellular GSH, allowing the recovery of its constituent aminoacids by the cell. A continuous GSH efflux in fact is present in most cell types, and thus sort of a "GSH cycling" across cell membranes takes place. In this perspective, GGT occupies a central position in cellular homeostasis of GSH and sulfur-containing aminoacids, and as such it is part of the 'antioxidant-antitoxic' defenses of the cell. However, the GGT-mediated catabolism of GSH also leads to the production of prooxidant species (superoxide, H_2O_2, thiyl radicals), as a result of the interaction of GSH metabolites with trace levels of transition metal ions. GGT/GSH-mediated prooxidant reactions produce the oxidation of SH groups in proteins of the cell surface, as well as the S-thiolation of cellular proteins. These reactions were shown to produce a significant stimulation of NF-kB nuclear translocation, as well as a stimulation of AP-1 DNA binding. Further studies have evidenced redox effects on the protein-kinase/phosphatase balance and on -SH groups of the TNF-α receptor TNFR1. The implications of these observations appear of importance. As documented in a series of studies, GGT/GSH-mediated prooxidant reactions do occur and can have a pathogenic role in a number of diverse conditions, such as rat liver chemical carcinogenesis, LDL oxidation and human atherosclerosis, tumor cell proliferation and apoptosis, resistance of tumor cells to cisplatin and ischemic kidney damage.

Nitrosopropofol and dipropofol: two nitrosoglutathione derivatives active on the mitochondrial energy metabolism

Roberto Stevanato, Sabrina Fabris, and **Federico Momo**

Department of Physical Chemistry, University of Venice, Dorsoduro 2137, 30123 Venice, Italy

In isolated rat liver mitochondria, the simultaneous presence of both the physiological NO donor nitrosoglutathione (GSNO) and the general anaesthetic propofol (2,6-diisopropylphenol) gives rise to a synergic action in affecting the resting and the ADP-stimulated respiration, the respiratory control ratio, the ATP synthesis and the formation and utilization of the electrochemical transmembrane potential. The *in vitro* reaction between GSNO and propofol, in the presence of Cu(II),

gives 2,6-diisopropyl-4-nitrosophenol (DPPNO) and 4,4′-dihydroxy-3,3′,5,5′-tetraisopropylbiphenyl (dipropofol) which we synthesized to verify their possible influence on the mitochondrial respiration.

The presence of DPPNO in the incubation medium of respiring mitochondria interferes with both the oxidative phosphorylation and the electron transport. The experiments suggested that the major effect of DPPNO is referable to an alteration of the mitochondrial membrane structures and that, compared to propofol, the molecular modification due to introduction of the NO group might play a critical role. In fact, DPPNO shows a pKa value of about 7.5 with respect to a value of about 11 for propofol, indicating that at physiological pH DPPNO is much more dissociated than propofol. Furthermore, DPPNO appears to be less hydrophobic than DPP as it results by measurements of octanol-water and liposomes-water partition coefficient. In fact both molecules are highly lipophylic and adsorb almost entirely in the lipid phase, but while the effects of DPP on the lipid organisation are quite similar to phenol, DPPNO is a stronger perturbing agent which accumulate into the interfacial region of the bilayer. Analogous investigations for dipropofol are in progress, in consideration of its possible estrogenic and redox properties.

Genetic polymorphism in the glutathione S-transferase supergene family: implications for susceptability and outcome in complex diseases

Richard C. Strange and **Anthony A. Fryer**

Clinical Biochemistry Research Laboratory, University Hospital of North Staffordshire, Hartshill, Stoke-on-Trent, England, ST4 7PA

The glutathione S-transferase supergene family encodes cytosolic and membrane-associated enzymes that demonstrate a very wide range of catalytic activities. GST enzymes appear to play a critical role in the protection of cells from the toxic actions of environmental and endogenous chemicals including byproducts of oxidative stress. The cytosolic group of GST is encoded by genes grouped into the Alpha, Kappa, Mu, Omega, Pi Sigma, Theta and Zeta classes. Genetic polymorphism has been identified in GST genes and many studies have attempted to identify associations between allelic variants and disease risk and outcome. For example, initial studies attempted to show that homozygosity for the null allele at GSTM1 was associated with an increased risk of lung cancer. Subsequently, researchers have investigated the clinical consequences of polymorphisms in GSTT1, GSTM3 and GSTA1/A2. Recently described polymorphisms in the Omega class GST are also being assessed for links with clinical phenotype. Many early studies were based on small study groups and, consequently there was considerable inter-study discrepancy in reported associations. However, more recent meta analyses of published data have shown convincing associations between GST polymorphisms and disease risk. Further there are now published studies confirming previously observed associations. For example we have reported associations between allelic variants of GSTP1 and asthma phenotypes in several study groups. Overall, therefore, there is convincing evidence that polymorphism in GST genes does affect clinical phenotype in several pathologies.

Glutathione transferases and the evolutionary redesign of their substrate selectivities

Bengt Mannervik

Department of Biochemistry, Uppsala University, BMC, Box 576, Uppsala, Sweden

Glutathione transferases (GSTs) have activity with different electrophiles and the numerous enzymes are distinguished by characteristic substrate selectivities. Natural evolution has effected the development of GST actives with several noxious products of oxidative stress, e.g., 4-hydroxynonenal, o-quinones, and epoxides. The GSTs therefore serve in the cellular protection against cancer and several other degenerative diseases. GSTs have previously been redesigned by rational structure-based approaches. Relative selectivities against alternative substrates have been changed by several orders of magnitude. Alternatively, GSTs with novel activity profiles can be obtained by mining the multidimensional functional space of a library of mutant enzymes. Multivariate statistical methods were used to characterize a glutathione transferase T1/T2 library constructed by stochastic recombinations of parental GST T1-1 and GST T2-2 DNA sequences, and subgroups of functionally similar variants were identified. The mutant enzymes were functionally characterized by use of six alternative electrophilic substrates representing different types of chemical reaction and diverse molecular shapes. Statistical cluster analysis divided the sampled clones into functionally related subgroups of GST variants. Members of these subgroups were selected as parents for a new generation of recombinant GSTs with enhanced catalytic efficiency with alkylhalogenides. 13 in a sample of 1000 mutants in the new generation displayed markedly enhanced activity with the targeted substrate. One of the mutants was purified and compared with wild-type GST T1-1. Its catalytic efficiency was enhanced by 100-fold. In this case nucleophilic substitution in alkyl transfer reactions was the activity screened for. In an alternative evolutionary pathway addition reactions may be targeted. Multivariate cluster analysis is a powerful tool to optimize the selection of parental molecular progenitors for the next generation of improved catalysts. Thus, protein engineering can complement Nature in the tailoring of GSTs for novel functions.

Nitrosylation of human glutathione transferase P1-1 with dinitrosyl-diglutathionyl iron complex *in vivo*

E. Cesareo[1], **J. Z. Pedersen**[1], **M. Nuccetelli**[1], **A. P. Mazzetti**[1], **G. Federici**[2], **G. Ricci**[1], and **M. Lo Bello**[1]

[1] Departments of Biology, University of Rome ''Tor Vergata'', 00133 Rome, Italy
[2] The Children's Hospital IRCCS ''Bambino Gesù'' 00165, Rome, Italy

Glutathione transferases (GSTs) are a family of enzymes involved in cellular detoxification from toxic and carcinogenic substances trough the conjugation of the sulfur atom of glutathione to an electrophilic center of these compounds. In this study we suggest a new and interesting role for GST P1-1 in the detoxification of cells from nitric oxide. Our previous *in vitro* studies demonstrated that dinitrosyl-diglutathionyl iron complex (DNDGIC), NO-donor in biological systems, binds with extraordinary affinity to human GST P1-1 suggesting that this enzyme may act as a NO carrier or scavenger. On the basis of these findings we tested the hypothesis that the active site Tyr7 residue may be responsible for the binding of DNDGIC, by producing three mutants of GST P1-1: Tyr7Phe, Tyr7His and Tyr7Cys. Kinetic characterization and EPR analysis of all these purified mutant enzymes showed about 10^{-4} times lower binding affinities for all three mutants giving evidence that the active site Tyr 7 is involved in the coordination of the iron atom of DNDGIC complex. As a part of this work we studied the formation and binding of the DNDGIC in *E. coli* cells overproducing GST P1-1, upon incubation with GSNO. EPR analysis showed the presence of a characteristic EPR signal of a protein-bound dinitrosyl-dithiol iron complex ($g = 2.03$) at room temperature and significant inhibition of GST activity. These results indicate that bacterial cells, in response to NO treatment, are able to form DNDGIC complexes, using intracellular iron and GSH. This complex may survive when stabilized through binding to GST P1-1.

Metabolism and Nutrition

Molecular mechanism by which glutamine stimulates the argininosuccinate synthetase gene expression

C. Brasse-Lagnel, A. Lavoinne, A. Fairand, and **A. Husson**

ADEN, EA 3234, IFR no 23, Faculty of Medecine, Rouen, France

We previously observed that the expression of the gene of argininosuccinate synthetase (ASS), the rate-limiting enzyme of arginine synthesis, is stimulated by the addition of glutamine to Caco-2 cells. In this work, the mechanism of action of the amino acid was further explored and the effect of glucosamine was studied in order to determine whether the hexosamine pathway was involved.

Culturing Caco-2 cells in the presence of 10 mM glutamine for 20 h induced a significant increase in both mRNA level ($174 \pm 26\%$; $n = 5$; $p < 0.05$) and enzyme activity ($190 \pm 21\%$; $n = 5$; $p < 0.05$). Similarly, 10 mM glucosamine produced an increase in both mRNA level ($227 \pm 36\%$; $n = 4$; $p < 0.05$) and enzyme activity ($239 \pm 30\%$; $n = 4$; $p < 0.05$). This was related to a two-fold increase in the transcription rate of the *ASS* gene, and the transcription factors potentially involved were searched by using EMSA experiments. Using the Sp1 consensus sequence or a fragment of the *ASS* gene promoter, both glutamine and glucosamine were shown to stimulate the DNA-binding of Sp1. Immunoprecipitation with Sp1 antibodies followed by Western blot with anti-O-linked *N*-acetylglucosamine showed that both glutamine and glucosamine increased the glycosylation status of Sp1. The stimulating effect of glutamine on enzyme activity, Sp1-DNA-binding and glycosylation, but not that of glucosamine, was totally suppressed by the addition of 40 μM 6-diazo-5-oxo-L-norleucine (DON), an inhibitor of glutamine amidotransferases. Altogether, these results demonstrate that glutamine metabolism through the hexosamine pathway is required to stimulate the ASS gene expression in Caco-2 cells, by increasing Sp1 glycosylation and, therefore, its DNA-binding capacity.

Assessment of folate status: measurement of homocysteine versus vitamin B12 and folate

Bakhouche Houcher[1], **Mirande Candito**[2], **Pierre Gibelin**[3], **Anne Caramella**[4], **Geneviève Poitier de Courcy**[5], **Dalila Naimi**[6], **Jean-Claude Lefebvre**[4], and **Emmanuel van Obberghen**[2]

[1] Département de Biologie, Faculté des Sciences, Université de Sétif, Sétif 19000, Algérie
[2] UI 145 Laboratory of Biochemistry,
[3] Department of Cardiology and
[4] Laboratory of Virology, Hôpital Pasteur, 30 Avenue de la Voie Romaine-B.P. 69, 06002-Nice Cédex 1, France
[5] Nutrition and Alimentation Scientific Institut, CNAM, 75141 Paris Cedex 3, France
[6] Département de Biologie, Faculté des Sciences, Université Mentouri, Constantine 25000, Algérie

This study was based on 63 subjects (mean age 50 ± 9) including 41 patients with clinical and objective investigational evidence of vascular disease (age 30–66 years) and 22 control subjects (age 26–54 years). Dietary intake of folate was estimated from a food questionnaire of folate (*Q*. folate). There was no statistically significant difference between controls and patients for dietary folate. By contrast, the folate level did not differ significantly between the control and patient subjects (6.68 ± 2.52 ng/mL versus 5.62 ± 4.16 ng/mL), respectively. Patients had higher average homocysteine concentrations than control subjects (12.93 ± 8.79 vs 9.07 ± 3.08 μmol/l, $P < 0.05$). A significant relationship was observed in controls between plasma folate concentrations and the homocysteine levels ($rs = -0.538$; $P < 0.01$), and the folate questionnaire ($rs = 0.697$; $P < 0.001$). We found in controls significant positive correlation between vitamin B12 and vitamin B6 ($rs = 0.475$, $P < 0.05$). By contrast, no association was found in patients between folate concentrations and the homocysteine levels and the and the dietary nutrient intake of folate. On the other hand vitamin B12 is inversely associated with homocysteine ($rs = -0.333$, $P < 0.05$). These data provide further evidence that plasma homocysteine is an independent risk factor for myocardial infraction. Homocysteine is a valuable functional index of micronutrient status and intakes for people, which can assist the development of the public health. In our population, folate was the most important determinant of plasma homocysteine, even in subjects with apparently adequate nutritional status of this vitamin.

Neural tube defects and decreased vitamin B12 values. Study of two cases in Algeria and one case in France

Mirande Candito[1], **Bakhouche Houcher**[2], **Catherine Boisson**[3], **Jacques Abellard**[4], **Marie-Joelle Demarcq**[5], **Jean-Louis Guéant**[6], **Khaled Benhacine**[7], and **Emmanuel Van Obberghen**[1]

[1] UI 145 and Biochemistry, Hospital Pasteur, 30 av de la Voie Romaine 06002 Nice Cedex 1, France
[2] Biology, Faculty of Sciences, Université, setif, 19000, Algerie
[3] Biochemistry, Hôpital Croix Rousse, 69317 Lyon, France
[4] Gynécology-Obstetrics, and
[5] Biochemistry, H G, 07205 Aubenas, France
[6] Inserm 00-14, Biochemistry of Proteins, Hopital de Brabois, 54511 Vandoeuvre les Nancy, France
[7] Neuro-Surgery, CHU, Setif, 19000, Algerie

Periconceptional folic acid supplementation reduces the frequency of embryological neural tube defects (NTD). This fact led the USA to fortify grain products with folic acid (140 μg/100 g) starting in January 1998, with a resultant decrease in the incidence of NTD. Folate deficiency thus appears to be a risk factor for NTD. A deficiency in vitamin B12 (cobalamin) might be another risk factor. We investigated three women who conceived a fetus with NTD; no folate deficiency was found in these women but all three had decreased vitamin B12 levels. Samples were obtained from two women in Algeria, respectively 2 and 3 days after interruption of pregnancy, and from a woman in France a vegetarian, after one month after interruption of pregnancy. Red cell folate and plasma folate, vitamin B12, B6 and homocysteine were assayed and the mutations A677T, A2756G and A66G were sought. Elevated plasma folate levels were found in both Algerian women. Vitamin B12 levels in all three women were decreased or in the lowest quartile of normal values for healthy women in our laboratory and for women with normal pregnancy. One woman presented simultaneously a B12 deficiency, and three heterozygous mutations: mutation C677T in the metylenetetrahydrofolate reductase gene (the substrate is folate), mutation A2756G in the methionine synthase gene and mutation A66G in the methionine synthase reductase gene (in both the co-factor is vitamin B12), while no control woman had this profile, and another woman presented a A66G homozygous mutation. Folate and vitamin B12 metabolisms are linked. In vitamin B12 deficiency, circulating plasma folate is increased but the

red cell folate may be decreased. It would therefore be advisable to consider fortifying grain products both folic acid and vitamin B12.

Two homocystinurics with deficient CBS activity: additive mutations in vascular disease?

Mirande Candito, Pierre Dellamonica, Paul Hofman, Didier Jambou, and **Emmanuel Van Obberghen**

UI145, Laboratory of Biochimie, Laboratoire d'Anatomo-pathologie, Hôpital Pasteur 30, Avenue de la Voie Romaine 06001 Nice, Italy
Laboratoire d'Hématologie, Hôpital de Cimiez, 4 ave Victoria, 06003 Nice, France

Only one third to one half of all homocystinuric patients suffer thromboembolic accidents. In addition to the mutations identified in the cystathione β-synthase (CBS) gene, several other mutations are putative risk factors for vascular disease. We investigated two homocystinuric patients, both of whom had reduced CBS activity. One patient has never suffered from thrombosis while the second patient developed a venous thrombophlebitis before the age of 20 years. So we looked at an additional genetic risk factor, among those described in literature as factor V Leiden, the principal cause of familial venous thrombosis, as the G20210A mutation in the prothrombin gene, and as the C677T mutation in the methylenetetrahydrofolate reductase gene. Additionally, the 844ins68 mutation in the CBS gene and the A2756G mutation in the methionine synthase gene were screened.

In the patient with a venous thrombotic event, factor V Leiden and G20210A mutation were absent as the homozygous C677T mutation. At the age of 40 years, this patient suffered a muscular myolysis, was dead of pulmonary embolism, and autopsy failed to demonstrate atherosclerosis of the carotid or large coronary arteries. As noted by several authors, hyperhomocysteinemia could be a venous thrombosis risk factor, and, without additive factor, did not lead to atherosclerosis in our patient.

Pharma-nutritional applications of some amino acids

Antonio Casini

Dipartimento di Studi di Chimica e Tecnologia delle Sostanze Biologicamente Attive – Faculty of Pharmacy – University of Rome ''La Sapienza'' – P.le A. Moro 5, 00185 Roma, Italy

The old distinction between essential or not-essential aminoacids has lost little of consensus recently. Actually, nutritional requirements of aminoacids, for an efficient tissue synthesis in healthy individuals, take in account not-essential aminoacids also. This is more true in the presence of certain clinical conditions, characterized by high tissue catabolism, such as post-surgical events, traumas or burns. In these conditions some metabolic pathways concerning aminoacid biosynthesis might be impaired, while the effective requirement of someones strongly increase, due to the needs of tissue repair. These metabolic stresses can be counteracted by adequate nutritional supply and, in this context, supplements of particular aminoacids are useful either as nutrients or as therapeutic agents. Several experimental and clinical studies showed that supplementation of particular aminoacids, such as glutamine, arginine, ornithine, tyrosine, cysteine, taurine and, for some specific pathologies, BCAA, in calibrated doses, can improve the efficacy of a therapy, since some of these nutrients are active in stimulation of protein synthesis, or metabolism regulation, in immunitary processes, in reducing tissue depletion while improving the muscle trophism or other.

This resume is, obviously, generic and incomplete. However, since the reported results on the above aminoacids are sometimes discordant or not consistent, an attempt will be made with the aim to review the present knowledge on some important issues concerning aminoacids supplementation.

The relationship between plasma cholesterol, amino acids and acute phase proteins in sepsis

Carlo Chiarla, Ivo Giovannini, Felice Giuliante, and **John H. Siegel**

IASI-CNR Center for Pathophysiology of Shock, Department of Surgery, Hepatobiliary Unit, Surgical Intensive Care, Catholic University School of Medicine, Roma, Italy and Department of Surgery, UMDNJ, Newark, USA

Purpose of the study was to correlate degree of hypocholesterolemia to changes in plasma amino acids and in other metabolic variables in 8 severely injured septic patients. Measurement included plasma cholesterol, amino-acidograms, acute phase proteins, complementary variables and blood cell counts. The Fischer plasma molar amino acid ratio (leucine + isoleucine + valine)/(phenylalanine + tyrosine) was calculated. Plasma cholesterol for all measurements ($n = 145$) was 3.1 ± 1.1 mmol/L and, upon entry in the study, was correlated inversely with sepsis severity score ($p < 0.05$). Cholesterol was related directly to the Fischer ratio and to cystathionine, methionine and glycine ($r^2 = 0.36$ to 0.15, $p < 0.001$ for all). Besides, it was related directly to transferrin and alkaline phosphatase (accounting for the eventual effect of cholestasis), and inversely to C-reactive protein, fibrinogen, lactate and white blood cell count ($p < 0.001$ for all). Changes in Fischer ratio, cystathionine and alkaline phosphatase accounted for 82% of the variability of cholesterol: Cholesterol $= 31.3 + 6.9$[Fischer ratio] $+ 1.5$[cystathionine] $+$ 0.2[alkaline phosphatase] ($r^2 = 0.82$ $p < 0.001$). The single best correlate of cholesterol, among other metabolic variables, was C-reactive protein ($r^2 = 0.70$, $p < 0.001$). These data show that severity of hypocholesterolemia in sepsis is quantifiably related to changes in plasma amino acids, and to severity of acute phase response and metabolic decompensation. More study is needed to understand whether hypocholesterolemia in sepsis has only diagnostic or prognostic implications, or it may contribute to worsening of the disease.

Influence of microwave treatment on the D-amino acid content of meat

János Csapó, Gabriella Pohn, Zsuzsanna Csapó-Kiss, and **Éva Varga-Visi**

University of Kaposvár, Faculty of Animal Sciences, Department of Biochemistry and Foodstuff Chemistry, H-7400 Kaposvár, Guba S. u. 40, Hungary

In the scientific literature, much data can be found on the effect of the microwave treatment on the D-amino acid content of dairy products, but relatively little dealt with the meat based food in this respect. The researches were usually based on the combination of time and efficiency. That is why we researched the change of the D-aspartic (D-Asp) acid and D-glutamic acid (D-Glu) content of meat cakes during microwave heat treatment. The samples were treated by 750 W energy in the microwave for 2, 5, 10, 15 and 20 minutes, then following their homogenization the D-Asp and D-Glu content (the most frequently occuring amino acids in the biggest amount in meat proteins) of the samples after OPA/TATG derivatization were determined by a high performance liquid chromatography. Parallel with this the researches were carried out from the outer skin and from the inner parts of the meat cake as well.

We found the proportion of the D/L Asp 0,048, the D/L Glu 0,027 in the untreated meat cakes, which can be explained at a great chance by the protein hydrolyses, the derivatization, and the racemisation taken place during the determination of the D-AA. Under the effect of the two minutes microwave treatment, this proportion grew 0,070 in the case of Asp and 0,038 concerning Glu, then both of them increased at a slight amount until the 10 minutes treatment. After 10 minutes the amount of both D-AA is increased significantly: until the 20th minute the D/L Asp ratio grew to 0,151 and the D/L Glu to 0,047. It is shown by the researches that the racemisation of the Asp is more considerable than the Glu and also that in case of the 10 minutes treatment 15% of the Asp, and 4–5% of the Glu can alter to D-enantiomer. In case of the 10 minutes there was no significant change concerning the D-AA content of the inner and outer parts of the meat cake, however, following this the shell of the meat cake contained significantly more D-Asp and slightly more D-Glu than the inner parts. From the researches we can take into consideration the conclusion that on the impact of 10 minutes microwave treatment used in households no significant changes are experienced in the D-AA content, while after that due to the effect of a longer microwave treatment the amount of D-amino acids can be considerable.

(Keywords: Microwave heating – D-aspartic acid – D-Asp – D-glutamic acid – D-Glu – D-amino acids – Racemisation of amino acids.)

Changing the free D-amino acid content of different type of cheeses influenced by the ripening period

János Csapó, Éva Varga-Visi, Gabriella Pohn, and **Zsuzsanna Csapó-Kiss**

University of Kaposvár, Faculty of Animal Sciences, Department of Biochemistry and Foodstuff Chemistry, H-7400 Kaposvár, Guba S. u. 40 Hungary

The purpose of our research was to determine the variation in free D-aspartic acid (D-Asp), free D-glutamic acid (D-Glu) and free D-alanine (D-Ala) content of dry matter; the variation in free D-amino-acid (D-AA) composition; and that of the ratio of free D-AA related to total (D + L) free amino acid during experimental cheddar cheesemaking processes with two different starter strains (Lactococcus lactis subsp. cremoris 303 or Lactococcus lactis subsp. cremoris AM2). Until the ninth week of ripening, only the increase of free D-Ala content of dry matter was significant ($P < 0.05$). The intensity of processes which are connected with lysis and responsible for release of D-Asp and D-Glu from cell wall and cytoplazm do not seem to increase until this stage of the ripening cheddar. The dry matter of curd contained a significantly higher amount of D-Asp after pressing than before pressing in case of both strains ($P < 0.05$); and more D-Glu in case of strain ''303''. These differences probably cannot be attributed to lysis. During ripening, the ratio of D-enantiomer within the free Ala content did not change due to the increase of D-Ala content following the increase of L-Ala content. One might speculate that part of the free D-Ala content derived from the free L-Ala content of cheese due to the bacterial alanine racemase if this enzyme can operate outside of the bacterial cell wall. Cheeses from cheesemaking trials with Lactococcus lactis subsp. cremoris 303 contained more D-AA ($P < 0.01$) and the ratio of D-enantiomer within the total (D + L) free Glu and total free Ala was higher ($P < 0.01$) than in the case of trials with Lactococcus lactis subsp. cremoris AM2. It is possible that strain ''303'' is more susceptible to lysis than strain AM2 during this sort of Cheddar making protocol and therefore releases more D-AA from cells or/and released enzymes form more D-AA. The ratio of the individual D-AA related to the total amount of D-AA (the D-AA composition) changed during ripening ($P < 0.001$) but there were no significant differences between the two strains.

(Keywords: Cheddar cheesemaking process – D-aspartic acid – D-Asp – D-glutamic acid – D-Glu – D-alanine – D-Ala – D-enantiomer.)

Determination of the protein of bacterial origin from the digestive system of cocks based on D-amino acid (D-Asp, D-Glu, D-Ala) content of excreta

János Csapó[1], **János Schmidt**[2], **Gabriella Pohn**[1], **Éva Varga-Visi**[1], **Zsuzsanna Csapó-Kiss**[1], and **Anita Juhász**[2]

[1] University of Kaposvár, Faculty of Animal Sciences, Department of Biochemistry and Foodstuff Chemistry, H-7400 Kaposvár, Guba S. u. 40, Hungary

[2] West-Hungarian University, Faculty of Agricultural Sciences, H-9200 Mosonmagyaróvár, Vár 2, Hungary

During our research, we studied the protein content from bacterial origin from control and appendices freed roosters' defecation by means of bacterial markers (diamino-pimelic acid, DAPA; D-aspartic acid, D-Asp; D-glutamic acid, D-Glu and D-alanine, D-Ala). Comparing the markers we determined that besides DAPA, the D-Asp, the D-Glu and the D-Ala can be well used for measuring the amount of the bacterial protein synthesis taking place in the digestive system of the poultry. Analysing it by linear regression, the value of the r among the DAPA, the D-Asp, the D-Glu and the D-Ala is the following in order: 0,463, 0,467, 0,669.

We suggested that in the case of the appendices freed animals we only have to consider the protein synthesis in the colon. By the analysis of the mixed defecation of the intact and appendices freed chicken we wished to confirm directly the fact that there is an intense microbial protein synthesis in the appendices. Analysing the digestibility of corn, wheat, extracted soybean meal and fish meal, we determined the DAPA and the D-AA content of the intact and appendices freed chickens by ion exchange column chromatography and a high performance liquid chromatography, and calculated the amount of proteins synthesized by the microbes in the defecation.

We determined that the mixed defecation of the intact animals contained 71% more microbe protein than the appendices freed animals'. While the proportion of the microbe protein content of the row protein content in the defecation of the intact animals was 17.8%, under the effect of the appendices cutting this proportion lowered to 11.2% by nearly 40%. During the feeding of different fodders an average of 0,72 g microbe protein have been synthesized in the appendices (extremes 0,35–1,09 g) which is 8,6% of the outgoing protein with the defecation. The microbe protein situated in the defecation of the appendices freed animals can be related to the microbe activity in other parts of the alimentary canal.

(Keywords: Bacterial protein synthesis – Diamino-pimelic acid – D-aspartic acid – D-glutamic acid – D-alanin.)

Extracellular levels of amino acids and choline in human high grade gliomas: an intraoperative microdialysis study

L. Bianchi[1], **E. De Micheli**[2], **A. Bricolo**[2], **M. Fattori**[1], **C. Venturi**[1], **F. Pedata**[1], **K. F. Tipton**[3], and **L. Della Corte**[1]

[1] Dipartimento di Farmacologia Preclinica e Clinica ''M. Aiazzi Mancini'', Universitá di Firenze, Viale G. Pieraccini 6, 50139 Firenze, Italy

[2] Dipartimento di Neurochirurgia, Università di Verona, Italy

[3] Department of Biochemistry, Trinity College, Dublin 2, Ireland

Available information on the metabolism of brain malignant gliomas is scarce and mostly derived from studies on animal models. Human data are limited to studies that have employed positron emission tomog-

raphy and proton magnetic resonance spectroscopy, or analysis of tissue specimens obtained at surgery or autopsy. The concentrations of endogenous amino acids in the extracellular fluid of human cerebral gliomas have been measured, for the first time, by *in vivo* microdialysis. Amino acids were determined by fluorimetric detection of their *o*-phthalaldehyde derivatives following resolution by hplc. Choline was also determined, as an established marker of tumour growth. Glioblastoma growth was associated with increased concentrations of choline, GABA, isoleucine, leucine, lysine, phenylalanine, taurine, tyrosine and valine. There was no difference between grade III and grade IV tumours in the concentrations of phenylalanine, isoleucine, tyrosine, valine and lysine, whereas the concentrations of choline, aspartate, taurine, GABA, leucine and glutamate were significantly different in the two tumour-grade subgroups. In contrast to the other compounds, the concentration of glutamate was decreased in glioma. The parenchyma adjacent to the tumour showed significant changes only in the extracellular concentration of isoleucine and valine. The concentrations of glutamate, leucine, taurine and tyrosine showed significant positive correlations with the degree of cell proliferation. These results may have important implications for the prognostic monitoring of glial tumours in the human and assessment of therapeutic responses. Epilepsy, which is relatively common in subjects with gliomas, was shown to be a significant confounding variable when the extracellular concentrations of aspartate, glutamate and GABA were considered.

α-Methyl-L-tryptophan as a tracer to evaluate the brain serotonergic system

Mirko Diksic

Department of Neurology and Neurosurgery, and Montreal Neurological Institute, McGill University, Montreal, QC, Canada

The dysfunction of the brain serotonergic system has been implicated in many brain diseases and disorders (*e.g.*, depression, borderline personality disorder, Parkinson's and Alzheimer's diseases). We have developed a method to measure regional serotonin synthesis, using radioactively labelled ''-methyl-L-tryptophan and positron emission tomography in humans, and using autoradiography in small laboratory animals. The studies in laboratory animals are important because they allow for better control of the conditions under which the synthesis is measured, and a wider range of perturbation, which permits the assessment of the basic principals on which the method is based. We have found that labelled'' -MTrp trapping in the brain correlates with the brain conversion of tryptophan to serotonin, but not with tryptophan incorporation into brain proteins, and an inhibition of tryptophan hydroxylase with p-chlorophenylalanine (administered for three days before the measurement) decreases brain serotonin synthesis as measured by the trapping of -MTrp. In contrast to this, an inhibition of protein synthesis with cyclohexamide does not affect regional serotonin synthesis. Treatments with specific agonists for the 5-MT1A or 5-HT1B receptor sites acutely decrease 5-HT synthesis, but chronic treatments do not have a significant effect on regional 5-HT synthesis. In human studies, we have demonstrated that a reduction in the plasma tryptophan concentrations, the method extensively used in the study of human behaviour after lowering the body and plasma tryptophan, results in a significant reduction in brain 5-HT synthesis. The reduction was substantially greater in females (40 times) than males (ten times). We also reported that there are regional specific differences in 5-HT synthesis between the controls and the patients diagnosed with borderline personality disorder. This suggests that there are differences in the affected regions between male and female subjects. For the first time, an unbiased measurement of a presynaptic function, namely 5-HT synthesis, in the living human brain can be done. The influence of different drugs used in the treatment of affective disorders, on brain 5-HT synthesis, can be evaluated. The changes observed in 5-HT synthesis could also be correlated with behaviour changes produced by these treatments.

The protein "Royalisin" function on hormonal profile, pathology and histochemistry in alloxan-diabetic male rats

E. F. Eskander[1] and **Nabila H. Ahmed**[2]

[1] Department of Hormones and
[2] Basic Medical Science (Field of Pathology), National Research Centre, Dokki, Cairo, Egypt

Administration of royalisin protein to alloxan-diabetic male rats resulted in marked improvement in the physiology of different organs represented in: Highly significant decrease in serum glucose level. Significant decrease in each of serum AST, ALT, alkaline phosphatase and bilirubin. Highly significant decrease in serum cholesterol level. Significant decrease in both of serum urea and creatinine levels. Highly significant increase in serum testosterone level with concomitant decrease in serum acid phosphatase level. Histological and histochemical evaluation of the pancreas and liver of the royalisin protein-treated animals revealed marked progress in the architexture of these two organs represented in: Restoration of near normal architexture of pancreatic islets whileas the DNA content in cells retracted to normal state. The liver showed a dilated vascular tract with marked increase in Von Kupffer cells, moderate regulation in glycogen content and mild increase in the proliferated hepatic cells with normal DNA content.

Abnormalities in energy metabolism of individuals with vasospastic syndrome

Olga Golubnitschaja[1], **Kristina Yeghiazaryan**[1], and **Josef Flammer**[2]

[1] Department of Radiology, Division of Molecular/Experimental Radiology, University of Bonn, Germany
[2] Ophthalmological Hospital, University of Basel, Switzerland

Vasospastic syndrome (VS) is a phenomenon frequently observed especially in young European female population. These individuals exhibit a characteristically prolonged spastic reaction of peripheral vessels on stress conditions compared to non-vasospastic individuals. This effect experimentally provoked by cold stress can be well seen in individuals with VS using microscopic monitoring of peripheral vessels. Characteristic elevated level of endothelin-1 values in blood of vasospastic individuals indicates specific alterations in molecular mechanisms resulting in the abnormal vascular regulation that is especially pronounced under stress conditions. Although molecular mechanisms leading to vascular regulatory abnormalities are not clear yet, an observation that fasting enhances the vasospastic effect indicates a functional link between VS and energy metabolism. Recently we could show specific differential gene expression in mononuclear blood cells of patients with normal-tension glaucoma accompanied by VS. In this work we looked for a specific differential gene expression of vasospastic individuals vs. non-vasospastic controls using transcriptomics applied on total pools of transcripts isolated *ex vivo* from mononuclear blood cells. Sixty genes the function of which is relevant for vascular regulation were found to be differentially expressed in VS individuals, whereby 48 genes were up-regulated and 12 genes were down-regulated compared to the control group. Intriguing was a finding that the majority of suppressed genes has a function in the energy metabolism such as an adrenodoxin, alcohol-dehydrogenase 6 and a mitochondrial trifunctional protein enoyl-CoA-hydratase. We conclude, therefore, that the altered regulation of the energy metabolism might be a clue in abnormal molecular mechanisms characteristic for VS.

Placental cell activity in tumor-bearing rats submitted to leucine supplemented diet

M. C. C. Gomes-Marcondes, G. Ventrucci, M. T. Toledo, and **M. A. R. Mello**

Department of Physiology and Biophysics, State University of Campinas, UNICAMP, 13083-970, Brazil

Placenta is the fundamental structure to maintain fetal development. In growing cancer, host changes can harm the fetus welfare. Previous studies showed that branched chain amino acids supplementation could improve the tumor-bearing carcass condition. Pregnant Wistar-rats were distributed into: control, C; Walker tumor-bearing, W; pair-fed, pC; leucine supplemented diet, L; tumor-bearing rats submitted to leucine diet, WL; pair-fed with leucine diet pL groups. After 20 days of experiment, placental and fetal weights were registered and total placental protein and DNA as well as histological analysis were performed. The fetal/placental weight ratio was lower in W than WL group. The placenta protein content was significantly reduced only in W groups. No change in placenta DNA content occurred among the groups, although the proteinl/DNA ratio was decreased in W and WL groups. There was no change in placenta cell number in spite of reduction in cell size in W group. The histological analysis in both W and WL placenta showed edema and hemorrhagic points in the decidua and the spongytrophoblastic layers; the labyrinth layer presented lack definition of the vascular border. Despite of the reduced fetal and placental weights in the WL group, placental protein, DNA and total cell number were preserved, suggesting that leucine, acting as a cell signaling factor, was able to improve fetal development.

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Effects of leucine supplemented diet on protein metabolism in young tumor bearing rats

G. Ventrucci, M. A. R. Mello, and **M. C. C. Gomes-Marcondes**

Department of Physiology and Biophysics, State University of Campinas, São Paulo, 13083970, Brazil

Cachexia state impairs life quality and induces protein wasting which, in turn, leads to host death. The main objective of this study was to improve host carcass condition, offering branched chain amino acids excess in diet, on account of improving the cellular protein synthesis. Females Wistar rats (55 days-old) were distributed into: a non-pregnant group (V) and six pregnant groups: pregnant (P), tumour-bearing (PW), pair fed (Pp), and submitted to leucine supplemented diet (L), tumour-bearing leucine supplemented diet (LW) and pair fed submitted leucine diet (Lp). Walker tumour viable cells (2.5×10^5) were injected in tumour-bearing rats on 2nd day of pregnancy. After 20 days, the animals were sacrificed and gastrocnemious muscle protein synthesis [phenylalanine H^3 incorporation to protein] and degradation [tyrosin release] were measured and myosin quantified [Western Blot]. Muscle protein synthesis was decreased in both tumour-bearing groups, specially in PW rats ($V = 4.12 + 0.17\,\text{nmol} \cdot \mu\text{g protein}^{-1} \cdot \text{h}^{-1}$; $P = 4.56 + 0.21$; $PW = 2.39 + 0.09$; $Pp = 3.52 + 0.23$; $L = 5.63 + 0.15$; $LW = 3.36 + 0.11$; $Lp = 3.62 + 0.17$; $p < 0.001$). Muscle protein degradation was increased in tumour-bearing groups, more in PW than in LW rats ($V = 0.078 + 0.005\,\text{nmol} \cdot \mu\text{g}^{-1} \cdot \text{h}^{-1}$; $P = 0.125 + 0.005$; $PW = 0.169 + 0.003$; $Pp = 0.149 + 0.004$; $L = 0.109 + 0.004$; $LW = 0.150 + 0.005$; $LP = 0.133 + 0.007$; $p < 0.01$). Muscle myosin heavy chain was deeply reduced only in PW group. These results indicated leucine supplemented diet can improve protein synthesis as well as maintain the main protein in skeletal muscle (myosin). Financial support Fapesp, CNPq, FAEP-UNICAMP.

Homocysteine: a key player in cellular one-carbon metabolism in Down's syndrome

M. C. Gueli

University of Palermo, Department of Cell Biology and Development, Section of Biochemistry, Policlinico, Via del Vespro 129, 90127 Palermo, Italy

Down's syndrome (DS) is of interest for the study of neurodegeneration because DS people develop dementia and neuropathological symptoms of Alzheimer's disease. By analogy with phenylketonuria the progressive mental retardation in DS babies may also be of metabolic origin. The gene for cystathionine β-synthase (CBS) is located on chromosome 21 and is overexpressed in children with DS. The metabolism of sulfur amino acids could probably be the source of the toxic compound.

To determine the specific imbalances induced by CBS overexpression, plasma levels of total homocysteine (tHcy), methionine (Met), cystathionine (Cystat), cysteine (Cys), and total GSH were measured by HPLC in children with DS and were compared to those from healthy controls.

The children with DS had significantly altered plasma levels of each of the metabolites in the Met/tHcy pathway and also in the CBS-mediated transsulfuration pathway. Plasma Hcy in DS children was 70–76% of that of the healthy children and a marked decrease (-52%) was observed for plasma Met concentration. Plasma levels of Cystat and Cys were significantly increased, consistent with an increase in CBS activity. The reduced plasma GSH observed in the children with DS most likely reflects a situation-linked antioxidant response to chronic oxidative stress, resulting from Cu–Zn superoxide dismutase overexpression.

These preliminary results indicate that in DS children CBS overexpression indirectly deprives the methionine synthase of the tHcy, while, at the same time, it creates ''the methyl trap'' and supports the increase of the Cystat and Cys levels. It is noteworthy that CBS is continously and strongly expressed in the CNS of human embryos from the earliest stage studied. CBS also has another enzymatic activity, the production of H_2S from Cys. Hyperproduction of H_2S secondary to overexpression of the CBS gene in DS could thus induce alterations in the CNS brain. Further studies are necessary to verify these hypotheses in DS children.

The possible role of liver in immuno-enhancing effects of arginine

X. L. Ma, C. J. Guo, Z. H. Geng, and **J. J. Yang**

Department of Nutrition, Institute of Hygiene and Environmental Medicine, Tianjin 300050, China

We hypothesized that the immune-enhancing effect of arginine might be mediated partly by the liver. We examined the direct effect of arginine on T cell proliferation as measured by ^{3}H-TdR incorporation *in vitro* and no stimulating or inhibiting effect was found when the arginine concentration ranged from 200 mg/L$\sim$1000 mg/L in the culture medium. In the next step, we cultured primary rat hepatocytes in serum-free DMEM/F12 medium, supplemented with different concentrations of arginine and the supernatants were collected at 0, 24, 48, 72 hours. Then, the supernatants were further added to the mediums for rat splenocyte culture. The T cell proliferation, IL-2 activity, NK cell activity and intracellular $(Ca^{2+})_i$ were determined respectively. The results showed that the supernatants significantly increased the Con A stimulated splenocyte proliferation, IL-2 activity, NK cell activity and intracellular $(Ca^{2+})_i$. It was concluded that the immuno-enhancing effects of arginine may be mediated at least partly by hepatocyte secreted factors after stimulated by arginine.

The quality of dietary protein affects urea synthesis in rats

K. Hayase[1], **K. Tujioka**[2], and **H. Yokogoshi**[2]

[1] Department of Home Economics, Aichi University of Education, Kariya, Japan
[2] Laboratory of Nutritional Biochemistry, School of Food and Nutritional Sciences, The University of Shizuoka, Shizuoka, Japan

The purpose of this study was to find whether the concentration of N-acetylglutamate would regulate urea synthesis when the dietary protein quality was manipulated. Experiments were done on three groups of rats given diets containing 10 g of gluten, 10 g of casein, or 10 g of whole egg protein/100 g for 10 days. The urinary excretion of urea, the liver concentration and synthesis of N-acetylglutamate, the liver concentrations of glutamate, and the liver activity of N-acetylglutamate synthetase increased with the decrease in quality of dietary protein. The acetyl CoA concentration in liver did not differ among the groups. A reverse correlation was observed between the activities of urea cycle enzymes, the plasma concentration of arginine and urinary excretion of urea under these conditions. N-Acetylglutamate concentration in the liver were closely correlated with the excretion of urea. These results suggest that greater N-acetylglutamate concentration in the liver of rats, given the lower quality of protein, stimulate urea synthesis and that the concentration of glutamate and activity of N-acetylglutamate synthetase in the liver are at least partly related to the hepatic N-acetylglutamate synthesis.

Changes in protein and amino acid metabolism in rats with acute acidosis

M. Holeček, R. Šafránek, and **L. Šprongl**

Department of Physiology, Charles University, School of Medicine, Hradec Králové and University Hospital Motol, Prague, Czech Republic

Acidosis is considered a significant cause of protein wasting in various pathologic situations. As its effect is modulated by other abnormal conditions, it is difficult to separate out the effects solely due to acidosis. We evaluated the direct response to acidosis *in vivo*, in isolated perfused liver and incubated skeletal muscle in rats.

In the first study, rats were infused for 6 h with HCl resulting in blood pH of 7.3 or saline. The parameters of protein metabolism were evaluated using L-[1-^{14}C]leucine. In the second study, using recirculation and single pass technique with 4,5-[^{3}H]leucine, [1-^{14}C]leucine and [1-^{14}C]ketoisocaproate we compared the response of isolated perfused rat liver to perfusion with solution of pH 7.2 and 7.4. In the third study, soleus and extensor digitorum longus muscles were incubated in medium of pH 7.4 or 7.3. [1-^{14}C]leucine was used to estimate protein synthesis and leucine oxidation. Tyrosine release was used to estimate proteolysis.

Negative protein balance and an increase in whole-body proteolysis, protein synthesis, leucine oxidation and amino acid concentration in the blood were observed in rats with acidosis. In the liver perfused with solution of pH 7.2 we observed higher rates of proteolysis, protein synthesis, amino acid utilization, urea production and ketoisocaproate decarboxylation than in controls. Decrease in pH of incubation media had no effect on protein synthesis, proteolysis, leucine oxidation and/or release of amino acids by both types of skeletal muscle.

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Effect of diet on amino acid profile of longissimus of young bulls

G. Holló, J. Csapó, G. Pohn, É. Visi, J. Seregi, I. Holló, and **I. Repa**

University of Kaposvár, Kaposvár, Guba S. 40, H-7400, Hungary

The objective of this study was to investigate the effect of full fat linseed meal supplementation of diet on the amino acid profile of longissimus. Hungarian Grey and Holstein Friesian growing finishing bulls were fed with either grass silage and concentrate or maize silage and concentrate with and without linseed supplementation according to a 2×2 factorial experimental design ($n = 10$/group). Concentrates for groups of A and C were supplemented 20% linseed meal containing 40% linolenic acid fed in the last month of the growing-finishing period. The average slaughter weights were 512.4 ± 58.4 kg, after a 24 hr chilling longissimus samples were taken from the right half carcasses. The amino acid composition of longissimus was analysed by a LABOR MIM amino acid analyser, altogether 17 amino acids were identified and quantified. The data processing was made by SPSS 10.0. The breed significantly influenced only the histidine content of longissimus. However, some amino acids (Ser, Pro, Gly, Cys, Val, Ileu, Leu, Phe) presented significant differences ($P < 0.05$) between groups fed with/without linseed supplementation rich in n-3 fatty acids. Breed and diet interaction can be established in case of histidine and the above-mentioned amino acids except valine. The differences in the fatty acid composition of diet appears to resulte in variation of the amino acid composition of longissimus.

Amino acid composition and biological value of beef as affected by gender

G. Holló[1], **J. Csapó**[1], **G. Pohn**[1], **É. Visi**[1], **E. Szücs**[2], **J. Tözsér**[2], **I. Holló**[1], and **I. Repa**[1]

[1] University of Kaposvár, Kaposvár, H-7400, Hungary
[2] Szent István University, Gödöllö, H-2103, Hungary

The influence of gender on amino acid and biological value of beef was compared using 12 male and 15 female Holstein-Friesian fattening cattle. Animals were fed with maize-silage, grass-hay and mixed-grain. Average slaughter weights for male and female animals were $463 + 25$ kg and $458 + 23$ kg, respectively. After chilling for 24 h, samples were taken from right half carcasses, between the 11–13th ribs. The amino acid profile of samples was made by LABOR MIM amino acid analyser, protein was hydrolysed in 6 mol hydrochloric acid for 24 h at 110°C. 17 amino acids were identified and quantified (Arg, His, Ile, Leu, Lys, Met, Tyr, Val, Ala, Asp, Glu, Gly, Pro, Ser, Tre, Cys, Phe). The biological value (BV) of protein was calculated by the 2:1 potato-egg mixture reference. Statistical analyses (SPSS 10.0) reveal significant differences between genders for the total amount of essential amino acids ($P < 0.05$), methionine ($P < 0.01$), isoleucine ($P < 0.001$), leucine ($P < 0.01$), lysine ($P < 0.01$), histidine ($P < 0.05$) and arginine ($P < 0.05$) but there was no significant effect for non-essential amino acids, and for the BV of proteins in meat. Gender may be a factor that significantly influences the amino acid profile in beef, as the quantity of essential amino acids was significantly larger in females than in males.

Protein-reduced rice, noodles and breads effectively enable amino acid scores perfect in low protein diets for chronic renal failure

Yoshie Kanazawa, Toshiyuki Nakao, Hiroshi Matsumoto, Tomonari Okada, Yume Nagaoaka, Hideaki Iwasawa, Ryo Tomaru, and **Toshikazu Wada**

Department of Nephrology, Tokyo Medical University, Tokyo, Japan

Low protein diet (LPD) is commonly used in the treatment for patients with chronic renal failure (CRF) and can effectively suppress the production of nitrogenous uremic toxins leading to prevent the emergence of uremia. In this diet, valid composition of amino acids (AA) is extremely important for the prevention of protein malnutrition. To simultaneously obtain both reduction of the amount and maintenance

of the quality of protein intake, foods that contain low AA score protein like rice, noodles and breads should be avoided. However, since sufficient energy intake is essential in the LPD therapy, these foods are necessary. In order to solve this dilemma in LPD, we tried to use specially manufactured protein-reduced rice, noodles and breads. The protein content of 100 g of these specialized foods compared with their conventional counterparts are as follows: protein reduced-rice, 0.16 g vs. 2.5 g; protein-reduced spaghetti, 0.6 g vs. 13.0 g; protein-reduced udon noodle, 2.7 g vs. 8.5 g; protein-reduced bread, 4.2 g vs. 9.3 g; protein-reduced croissant; 3.8 g vs. 7.9 g.

The dietary compositions of amino acids were studied in 22 patients with chronic renal failure on LPD. Dietary intake was calculated from precisely weighed food records during a period of 2 days. Validity of dietary AA composition was judged from AA score (AA-S) proposed by FAO/WHO.

Actual daily intake of protein in the patients was 0.61 ± 0.08 g/kg, energy 32.9 ± 3.5 kcal/kg, essential AA 13.0 ± 5.5 g, total nitrogen 5.4 ± 1.6 g. Mean AA-S was 94.1 ± 4.4, and the scores of 10 patients were 100 (group □) and those of 12 patients were below 100 (group □). There was no significant difference in protein intake between two groups. The percentage ratio of essential AA/protein intake was significantly higher in group□than in group □ (0.53 ± 0.06 vs. 0.38 ± 0.10, $p < 0.05$), and that of animal/vegetable protein was significantly higher in group□than group□ ($p < 0.05$). There was significant correlation between AA-S and the percentage ratio of essential AA/protein intake ($r = 0.557$, $p < 0.05$). The amounts and frequencies using protein-reduced rice, noodles and bread were significantly higher in the patients of group□than group □.

In conclusion, to maintain AA-S at 100 in LPD, over 60% of dietary protein should be composed of animal protein and the use of protein-reduced rice, noodles and breads is beneficial for achieve this.

Effect of sulforaphane on human lymphoblastoid cells

Irena Misiewicz[1], **Katarzyna Skupińska**[1], and **Teresa Kasprzycka-Guttman**[1,2]

[1] National Institute of Public Health, 00-725 Warsaw, Chelmska 30/34, Poland

[2] Department of Chemistry, Uniwersity of Warsaw, 02-093 Warsaw, Pasteura 1, Poland

Sulforaphane is naturally occurring isothiocyanate, which is mostly present in Cruciferous family. Both natural and synthetic sulforaphane seem to be very potent inducers of detoxification phase 2 enzymes and apoptosis. In this report, BRCA-1 $+/-$ human lymphoblastoid cells, established in the permanent cell culture by Epstein-Barr Virus immortalization of lymphocytes B originated from patient with germline mutation in one allele of BRCA-1 gene, were tested to evaluate sulforaphane as a potentially chemoprevntive agent. In this work, we studied the effect of sulforaphane on the induction of NAD(P)H:quinone reductase, GSH level, proliferation, levels of potooncogene c-myc and tumor suppressor BRCA-1 proteins, and on apoptosis. Quinone reductase activity was assessed using menadione/NADPH assay, a glutathione level was measured by Ellman's assay, a change in proliferation was estimated by MTT assay. Confocal Laser Scanning Microscopy was used to determine protein levels by immunofluorescence and to study apoptosis, using Annexin V assay. In our studies, sulforaphane was shown to influence qiunone reductase activity and stop cells growth. Treatment of cells with sulforaphane decreased the intracellular glutathione level, due to formation of dithiocarbamates. Higher concentration used, caused apoptosis. We also found correlation between a dose of sulforaphane and level of c-myc and BRCA-1 proteins. Our result strongly suggest, that sulforaphane should be further tested as potential carcinogenesis preventive agent.

Determination of the affinity of polycyclic aromatic hydrocarbons toward serum albumin

K. Skupińska[1], **E. Bojarska**[2], **I. Misiewicz**[1], and **T. Kasprzycka-Guttman**[1,3]

[1] National Institute of Public Health, Chelmska 30/34, 00-725 Warsaw, Poland

[2] Division of Biophysics, Department of Physics, Warsaw University, Zwirki i Wigury 93, 02-089 Warsaw, Poland

[3] Department of Chemistry, Warsaw University, Pasteura 1, 02-093 Warsaw, Poland

Polycyclic Aromatic Hydrocarbons are environmental pollutants resulting from incomplete combustion of an organic matter. Some of those compounds like benzopyrene are considered carcinogens. To assess risk of exposure to Polycyclic Aromatic Hydrocarbons biomarkers are used. Widely used ones are albumin- Polycyclic Aromatic Hydrocarbons adducts, since albumin is the most abundant protein in plasma. Association constants of Polycyclic Aromatic Hydrocarbons – albumin complexes were calculated. Four Polycyclic Aromatic Hydrocarbons were examined: anthracene and its three oxygen-containing derivates, that can be anthracene metabolites: 9,10-antraquinone, 1,5-dihydroxyantraquinone and 9-anthracenecarboxylic acid. The aim of the study was to establish if absorbed Polycyclic Aromatic Hydrocarbons can bind to albumin before metabolic activation and if kind and amount of functional groups influence affinity of binding. The constants were determined by measuring fluorescence quenching of single tryptophan residue in albumin, which is the effect of albumin–Polycyclic Aromatic Hydrocarbons interaction. Theoretical model used assumes that albumin has only one binding site available for each compound. Due to spectral properties of studied compounds the mathematical corrections was applied to calculate constants accurately, including inner filter effect and fluorescence of compounds. The calculated constants indicate, that only derivatives of anthracene bind effectively to albumin. Among all, 1,5 dihydroxyantraquinone, that has most oxygen group, was the most reactive. These results suggest that Polycyclic Aromatic Hydrocarbons must be first metabolized to bind to biological particles.

Diabetes mellitus is a risk factor for cardiac tissue degeneration

Heike Moenkemann[1], **An De Vriese**[2], **Henricus J. Blom**[3], and **Olga Golubnitschaja**[1]

[1] Department of Radiology, Division of Molecular/Experimental Radiology, University of Bonn, Germany

[2] Laboratory of Paediatrics and Neurology, University Medical Center Nijmegen, The Netherlands

[3] Renal Unit, Department of Internal Medicine, University Hospital, Gent, Belgium

Objective: Oxidative stress was proposed to be a critical factor in pathogenesis of diabetes mellitus (DM) and diabetic complications. The ethiology of cell degeneration in DM-induced cardiomyopathy are presently unclear. Transition between the apoptotic degeneration and cell proliferation under stress conditions is regulated at checkpoints of the cell cycle. The goal of this study was to investigate *ex vivo* expression of two oxidative stress responsible $p21^{WAF1/CIP1}$ and 14-3-3 σ genes combined with quantification of double-strand (ds)DNA breaks in cardiomyocytes of rats with streptozotocin-induced DM.

Results and conclusions: Whereas no detectable expression of 14-3-3 σ and only traces of $P21^{WAF1/CIP1}$ were found in control cardiomyocytes, constantly high expression rates of both genes were observed in the DM group. The measured level of dsDNA breaks was significantly lower in diabetic cardiomyocytes compared with controls. We proposed a dual role for the cell cycle regulation under diabetic conditions: both $p21^{WAF1/CIP1}$ and 14-3-3 σ genes are activated in order to trigger cell

cycle arrest and concomitant DNA repair that prevent the replication of mutated DNA. The double cell cycle arrest ultimately inhibits replication of cells which accumulate in the G_1 and G_2 phases, leading to retarded proliferative activity in diabetic myocardium.

D-Amino acids as respiratory substrates in microorganisms

Yoko Nagata[1], **Kazunari Abe**[1], **Yuichiro Hasegawa**[1], **Mika Ito**[1], **Takehisa Kawada**[1], **Minoru Tanigawa**[1], and **Kumiko Nagata**[2]

[1] Department of Materials and Applied Chemistry, College of Science and Technology, Nihon University, Tokyo 101-8308, Japan
[2] Department of Bacteriology, Hyogo College of Medicine, Hyogo 663-8501, Japan

D-Amino acid dehydrogenase catalyzes the degradation of D-amino acids and the production of hydrogen from the amino acids. Electrons generated from hydrogens reduce cytochromes in an electron transfer system. Peaks in the absorption spectra of cytochromes increase in height when the cytochrome is reduced. Therefore, an increase in the height of the absorption peaks of a cytochrome indicates that the cytochrome has been reduced, and that hydrogen has been supplied to the cytochrome in the electron transfer system.

In the present study, a D-amino acid dehydrogenase fraction that contaied no cytochromes, and a cytochrome *bo* fraction free from D-amino acid dehydrogenase were prepared by treatments with Triton X-100 from *Escherichia coli*. In the presence of the enzyme fraction, the difference absorption spectrum with and without D-alanine of the ctyochrome *bo* fraction showed the α- and γ-peaks at 563 nm and 430 nm, respectively. No oxygen uptake was observed in the cytochrome *bo* fraction in the presence of D-alanine. Upon addition of the enzyme fraction, oxygen consumption was observed. *Helicobacter pylori* is a bacterium associated with gastric inflammation and peptic ulcer diseases. *Pyrobaculum islandicum* is a hyperthermophilic archaeon whose optimum growth temperature is 100. Both of the crude extracts of these two microbes showed a similar change in the absorption peak to that of *E. coli* upon the addition of D-alanine, D-proline or D-valine. All of these results suggest the possibility that D-isomers of amino acids such as alanine, valine and proline are utilized by some microbes as respiratory substrates to produce ATP *via* electron transfer systems.

Release and uptake of amino acids across skeletal muscle in patients with chronic renal failure

Toshiyuki Nakao, Hiroshi Matsumoto, Tomonari Okada, Yume Nagaoaka, Hideaki Iwasawa, Ryo Tomaru, and **Toshikazu Wada**

Department of Nephrology, Tokyo Medical University, Tokyo, Japan

Protein-malnutrition and wasting is frequently seen and contributes to mortality and morbidity in patients with chronic renal failure (CRF). Abnormalities in plasma and muscle intracellular concentrations of free amino acids (AA) have been recognized in such cases. There is, however, little information about kinetics of AA, release and uptake, across skeletal muscle in CRF. This study attempted to gain insight into any alterations on release and uptake kinetics of AA across skeletal muscle in patients with CRF.

Six patients (4 men and 2 women) with CRF due to chronic glomerulonephritis, mean age 45.0 ± 16.6 years old, creatinine clearance 4.5 ± 2.8 ml/min, serum urea nitrogen 106.9 ± 43.5 mg/dl, serum creatinine 12.3 ± 3.1 mg/dl, and five controls (2 men and 4 women), mean age 34.8 ± 9.1 years old, who had normal renal function were included in this study with informed consents. Forearm arterial and venous bloods were simultaneously obtained after overnight fasting and also at 60 minutes after ingesting 100 g of glucose, and concentrations of 18 AA (8 essential, 2 semiessential and 8 nonessential AA) were measured. As an index of release and uptake of AA across forearm skeletal muscle, arterial-venous differences (AVD) of the AA concentrations were calculated.

AVD of total AA in the overnight fasting state were $+436.3 \pm 186.2$ nmol/ml in controls and $+42.1 \pm 169.7$ nmol/ml in CRF patients, and at 60 minutes after ingesting 100 g of glucose values were $+107.1 \pm 61.6$ nmol/ml in controls and $+198.9 \pm 89.8$ nmol/ml in CRF patients. Changes in AVD after glucose ingestion were significantly smaller in CRF patients than in controls ($+156.4 \pm 172.0$ vs. -349.2 ± 173.9 nmol/ml, $p < 0.01$). There were no significant changes in the AVD of branched chain AA, Val + Leu + Ile, between the two groups resulting from glucose ingestion ($+7.5 \pm 24.1$ vs. -11.4 ± 41.1 nmol/ml, NS). Changes in AVD of glucogenic AA, Ala + Gln, by glucose ingestion were significantly smaller in CRF patients than in controls ($+28.9 \pm 34.5$ vs. -116.8 ± 76.3 nmol/ml, $p < 0.01$).

These results demonstrate that suppression of release and acceleration of uptake of AA by skeletal muscle after carbohydrate load are poorer in CRF patients and this might be one of the contributing factors of muscle wasting in CRF patients.

Effects of Japanese millet protein on lipid profile and diabetes in rats

N. Nishizawa[1], **D. Sato**[1], **Y. Miyakoshi, T. Nagasawa**[1], **Y. Ito**[1], **You-Young Choi**[1], **Wei Yi Min**[2], and **Myeong-Rak Choi**[3]

[1] Department of Agro-Bioscience, Faculty of Agric., Iwate Univ., Morioka, Iwate, Japan
[2] Institute of Food Sci. and Technol., CAAS, Beijing, China
[3] Yeosu Natl. Univ., Yosu, Korea

Millet has been used widely as human food. With the exception of our previous study, there has been no study on the effect of millet on physiologic function. The consumption of millet has increased in Japan because of the expectation of health benefits. We therefore have examined the effects of protein derived from millet on lipid metabolism and diabetes. When rats were fed a diet of 20% Japanese millet protein plus 1% cholesterol for 20 days, the plasma levels of total and LDL cholesterol were significantly lower than those of control. Then, when diabetic rats were fed a cholesterol-free diet of 20% Japanese millet protein for 20 days, plasma glucose levels were significantly lower and adiponectin and HDL cholesterol levels were significantly higher than those in animals fed a control diet. Taken together with our previous study on the effects on plasma HDL-cholesterol levels or liver injury, these results imply that millet cereals have multiple beneficial effects on health.

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Pertubation of critical metabolic processes may be associated with 3-hydroxynorvaline induced teratogenesis

H. C. Potgieter, R. Louw, F. H. van der Westhuizen, E. Erasmus, and **L. J. Mienie**

Department of Biochemistry, School of Chemistry and Biochemistry, University of Potchefstroom, Potchefstroom, South Africa

Perturbation of key metabolic processes may be strongly associated with embryonic dysmorphogenesis, caused by microbial xenobiotics. A diastereomeric synthetic mixture of the fungal, non-protein amino acid, 3-hydroxynorvaline (HNV), was teratogenic to chicken embryos. Employing chicken embryos in metabolic studies is problematic and the mouse embryo model was selected for this purpose. Pregnant female mice

received HNV by gavage on days 7, 8 and 9 of the pregnancy. Controls received saline. A high incidence of exencepahalon were recorded in mouse embryos on day 18 of their development. Urinary metabolic profiles (i.e. organic acids, acylcarnitines, amino acids) of control and HNV-treated mothers were assessed in 24-h aseptically collected urine samples. The effect of HNV on maternal hepatic and whole embryo serine hydroxymethyltransferase and the glycine cleavage complex were determined. DNA methylation status and the synthesis of DNA, S-adenosylmethionine and polyamines (putrescine, spermidine, spermine) were also assessed. Significant differences between control and HNV treated animals and embryos were demonstrated for nearly all of the measured parameters. GC-MS studies revealed that selected isomers of the HNV were rapidly metabolised and 2,3-Dihydroxypentanoic acid, a potential inhibitor of fatty acid catabolism, appeared to be the main product. Cell culture studies confirmed that HNV inhibited β-oxidation of fatty acids. HNV causes multiple metabolic perturbations in mothers and their embryos. This will adversely affect one-carbon and energy metabolism, including a number of down stream processes (i.e. DNA synthesis, DNA methylation, polyamine synthesis, etc.), highly dependent on the generation of sufficient one-carbon units and ATP and important prerequisites for proper closure of the neural tube during embryogenesis.

Spirulina protein, body growth and skeletal muscle protein metabolism in young rats

F. A. Voltarelli[1], **M. C. C. Gomes-Marcondes**[2], and **M. A. R. Mello**[1]

[1] Departamento de Educação Física, UNESP, Rio Claro and
[2] Departamento de Fisiologia e Biofísica, UNICAMP, Campinas, SP, Brazil

As we enter the new milenium, malnutrition remains an important factor impairing health and productivity of large populations. New alternative alimentary protein sources became a matter of generalized interest. The present study evaluates the effects of the blue green alga *spirulina* as the sole dietary source of protein on protein metabolism in skeletal muscle of weanling (28 days-old) male Wistar rats. A 17% protein *spirulina* [S] diet fed ad libitum was compared to a 17% protein casein [C] diet [AIN 93]. Total protein and DNA contents and protein synthesis [amount of L[U14-C] phenylalanine incorporated] and degradation [release of protein-bound tyrosine] were measured in the isolated soleus muscle. Myosin protein expression in the gastrocnemius muscle was also measured [Western-blot]. Muscle total protein [$C = 2.99 \pm 0.35$; $S = 2.75 \pm 0.42\,mg/100\,mg$] and DNA [$C = 0.084 \pm 0.014$; $S = 0.074 \pm 0.014\,mg/100\,mg$] contents were similar in both groups. Protein degradation [$C = 427.5 \pm 146.2$; $S = 476.7 \pm 181.9\,nmol/g.h$] did not differ between the two groups but protein synthesis [$C = 17.5 \pm 3.5$; $S = 25.2 \pm 6.8\,nmol/g.h$] was higher ($p < 0.05$, t test) in *spirulina* than in casein fed rats. Myosin data are still in analysis. Spirulina protein proved adequate to maintain body growth and skeletal muscle protein metabolism in young rats.

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Human milk banking: comparison between different pasteurisation temperatures on levels of protein sulfur amino acids

Carratù Brunella[1], **Ambruzzi Amalia Maria**[2], **Fedele Elena**[1], and **Sanzini Elisabetta**[1]

[1] Istituto Superiore di Sanità – Roma, Italy
[2] Ospedale Bambino Gesù – Roma, Italy

Human milk is frequently heat treated in hospitals to reduce bacterial contamination, particularly in banked milk. However pasteurisation may induce losses of some nutrients and may inactivate enzymes and immunologic factors. This study was part of a wider research to define the guidelines concerning human milk banking and heating treatment at two different temperatures, 62.5°C (Holder method) and 56.5°C (less destroying) for 30 minutes for safety use. In this work we examined the effects of technological process on sulfur aminoacids levels of human milk.. Sulfur aminoacids are essential, and they affect nutritional value of human milk proteins because of their low levels and their oxidative instability, occurring especially during thermal treatment.

The levels of cysteine and methionine were determined in protein idrolysate of untreated and both pasteurised milk samples of 20 mothers. Due to the lability of cysteine and methionine during acid hydrolysis, they were more accurately determined after oxidation to cysteic acid and methionine sulfhone by performic acid treatment. Then they were analyzed by HPLC with precolumn derivatization using 9-fluorenylmethyl chloroformate with both UV and fluorescence detection. The obtained data from raw untreated samples were used to evaluate the losses of protein nutritional value in relation to pasteurisation treatment. Moreover statistical analyses were performed to know interdividual variability of examined lactating women.

A hypothesis on the biochemical mechanism of tetrahydrobiopterin-responsiveness in phenylalanine hydroxylase deficiency

Robert Steinfeld[1] and **Zoltan Lukacs**[2]

[1] Department of Pediatrics, University of Goettingen and
[2] Department of Pediatrics, University of Hamburg, Germany

We have examined six children with tetrahydrobiopterin responsive phenylalanine hydroxylase deficiency. All patients carry two mutant alleles in the phenylalanine hydroxylase gene. Cofactor deficiency was excluded. The effect of tetrahydrobiopterin administration was studied by correlating different oral tetrahydrobiopterin doses with plasma phenylalanine levels under defined protein intake. Our results indicate that oral tetrahydrobiopterin supplementation may be used as long-term treatment for individuals with tetrahydrobiopterin -responsive phenylalanine hydroxylase deficiency, either without or in combination with a less restrictive diet.

Previous *in vitro* studies have demonstrated that tetrahydrobiopterin inhibits phenylalanine hydroxylase tetramers but activates phenylalanine hydroxylase dimers. To explain the beneficial effect of tetrahydrobiopterin supplementation, we hypothesize that the activity of phenylalanine hydroxylase mutants is stimulated by tetrahydrobiopterin in case the mutants predominantly form dimers. It may indicate, that tetrahydrobiopterin-responsiveness results from tetrahydrobiopterin induced stabilization of mutant phenylalanine hydroxylase dimers. In addition, interindividual differences in the cellular folding apparatus may determine the tertiary structure and the amount of mutant phenylalanine hydroxylase dimers and hence may account for divergent tetrahydrobiopterin-responsiveness reported for the same genotype.

Increased activity of metalloproteinases MMP-2 and MMP-9 in blood of patients with Diabetes mellitus as an indicator for the developing angiopathy

Kristina Yeghiazaryan[1], **Marko Kapalla**[2], **Marta Hricová**[2], **Antonín Pírek**[2], **Hans H. Schild**[1], and **Olga Golubnitschaja**[1]

[1] Department of Radiology, Division of Molecular/Experimental Radiology, University of Bonn, Germany
[2]Department of Clinical Biochemistry and Haematology, Alpha medical s.r.o., Ružomberok, Slovakia

Objective: An angiopathy is a frequent complication of both *Diabetes mellitus* (DM) type 1 and type 2. Thereby differential severity of the

angiopathy is characteristic for this group of patients. Although a severe form of angiopathy is a usual cause of further complications such as cardiomyo- and retinopathy, and even death, no reliable molecular markers have been established yet in order to prognose a grade of the angiopathy in individual DM-patients. Since both metalloproteinases MMP-2 and -9 have been shown to be implicated in an extracellular matrix degradation and remodelling of aortic tissue, we measured by zymography an activity of both MMPs in blood serum of individual DM-patients and sex and age matched non-diabetic individuals.

Results and conclusions: The activity of MMP-2 was independent from age and gender and was constantly increased for about two-times in DM compared to the non-diabetic group. This contrasted with the activity of MMP-9 which was varying significantly in both groups. In the DM-group this activity varied between the values which were equal and about four-times higher compared to an average in the non-diabetic group. This activity was sensitive to the level of glucose positive correlating with it, and gender dependent demonstrating higher levels in the male DM-subgroup. We conclude that 1. co-induced activity of both MMP-2 and -9 might indicate a developing diabetic angiopathy, and 2. the individual levels of MMP-9 activity in blood of DM-patients should be further taken into consideration as the positive marker to prognose a severity of the developing angiopathy.

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Modification of Amino Acids and Proteins

Peptide mass mapping and N-acetyl- and N-glycolyl-neuraminc acid analysis of pituitary purified bovine follicle stimulating hormone

V. Borromeo, A. Berrini, and **C. Secchi**

Department of Veterinary Pathology, Health and Hygiene – Biochemistry and Physiology Unit – University of Milan, 20133 Milan, Italy

Follicle stimulating hormone (FSH) is a pituitary glycoprotein composed of an α-subunit common to the gonadotropins and a hormone-specific β-subunit. The oligosaccharides constitute the major source of FSH isoform heterogeneity, however micro-heterogeneity in the peptide chains cannot be excluded. Our aim was to investigate the chemical basis of bovine FSH (bFSH) heterogeneity by searching for insertion, deletion or modification of amino acids and for presence of different types of sialic acids.

The purification of intact dimeric α/β bFSH was achieved by immunoaffinity chromatography based on a species-specific anti-β-subunit mAb. The primary structure was characterised by tryptic mapping and high-performance liquid chromatography with on-line electrospray ionisation mass spectrometry detection. The monosaccharides were analysed by high-performance anion-exchange chromatography with pulsed amperometric detection.

The peptide mass map showed only the expected fragments, except the peptide α1–15. However, the hypothesis of α-subunit NH_2-terminal heterogeneity was discarded by aminoacid sequencing of the first ten residues of intact bFSH. We concluded that the purified bFSH had no measurable primary structure modifications.

The carbohydrate accounted for 20.3% of the bFSH Mr, consisting of a mixture of fucose, *N*-acetyl galactosamine, *N*-acetyl glucosamine, mannose and galactose. Moreover, we detected equimolar ratio of two distinct sialic acids, *N*-acetyl- and *N*-glycolyl-neuraminc acid (Neu5Gc). This is the first evidence that both sialic acids are present on a mammalian pituitary glycoprotein hormone. The demonstration that bFSH carries significant amount of Neu5Gc remarks its species-specificity. In fact, Neu5Gc is absent in apes and man, due to the lack of a functional CMP-Neu5Ac-hydroxylase.

Acetylation of proteins as potential novel target for antitumor therapy

E. Di Gennaro[1], **G. Romano**[1], **M. Marra**[2], **F. Bruzzese**[1], **S. Pepe**[3], **M. Caraglia**[2], **A. Abbruzzese**[2], and **A. Budillon**[1]

[1] Department of Experimental Oncology, Istituto Nazionale Tumori-G. Pascale,

[2] Department of Biochemistry and Biophysics, Second University, Naples, and

[3]Department of Endocrinology and Mol. Clin. Oncology, University Federico II, Italy

The basic structure of chromatin is characterized, together with coiled DNA, by histones, whose postranslational modifications, such as acetylation, is implicated in regulation of gene expression. Histone deacetylase (HDAC) inhibitors have been recently shown to induce antiproliferative effect in a variety of human cancer cells by mechanism that cannot be solely attributed to the level of histone acetylation since deacetylation of other proteins rather than histones has been also described. We have evaluated if the HDAC inhibitor Suberoylanilide hydroxamic acid (SAHA) has potential applications as antitumor agent for colorectal cancer. We demonstrated that SAHA induced G1 cell cycle arrest, apoptosis and antiproliferative effect in colon cancer cells independently of p53 status. Interestingly, SAHA treatment led to reduced expression of mut-p53 and stabilization of wt-p53. Moreover, SAHA induced upregulation of expression and function of tissue transglutaminase, and enzyme implicated in post-translational modifications of proteins and in the regulation of apoptosis and of some signal transduction pathways. Furthermore, protein expression of thymidilate synthase, a critical target for chemotherapeutic agents active in colorectal cancer such as 5-fluorouracil (5FU) and Raltitrexed, was downmodulated by SAHA treatment. Finally, on the basis of this observations, we have demonstrated that the combination of SAHA and Raltitrexed or 5FU enhanced cell growth inhibition compared to single drug schedule. These results add new insights into the mechanism of antitumor action of SAHA, by defining part of the gene expression profile regulated by this molecule and suggesting that it should be further evaluated as antitumor agent for colorectal cancer.

Mono(adp-ribosylation) inhibition mobilises calcium from the endoplasmic reticulum by triggering a phospholipase c-like pathway: implications for apoptosis

Claudia Cerella[1], **Maria D'Alessio**[1], **Milena De Nicola**[1], **Simona Coppola**[1], **Maria Cristina Albertini**[3], **Augusto Accorsi**[3], **Antonio Bergamaschi**[2], **Andrea Magrini**[2], and **Lina Ghibelli**[1]

[1] Dipartimento di Biologia,

[2] Cattedra di Medicina del Lavoro, Universita' di Roma Tor Vergata, and

[3] Istituto di Chimica Biologica A. Fornaini, Universita' di Urbino, Italy

Protein post translational modification by mono(ADP-ribosylation) is involved in the regulation of signal transduction, as well as in intracellular apoptotic signaling. In this study we show that the inhibition of a mono (ADP-ribosylation) reaction, by 3-aminobenzamide (3-ABA) or nicotinamide, elicites an immediate (<15 seconds) Ca^{2+} mobilisation from endoplasmic reticulum (ER) in two cell lines of hematopoietic origin. The kinetic of the consequent Ca^{2+} fluxes differs from that induced by ER Ca^{2+}-ATPase poisoning (i.e., with thapsigargin), whereas it is identical to that induced by the phospholipase C (PLC)-mediated ER Ca^{2+} channel opening elicited by receptor stimulation. In line with this evidence, inhibition of PLC by neomycin or U73122 completely inhibited

3-ABA-induced Ca^{2+} mobilisation, suggesting that ADP-ribosylation inhibition immediately activates PLC-beta to stimulate Ca^{2+} fluxes from ER. We also found that PLC inhibition is able to reverse the strong effects that 3-ABA exerts on stress-induced apoptosis, that we previously described (i.e., increased rate of apoptosis and potentiation of plasma membrane blebbing, Ghibelli et al., 1995, *Exp. Cell Res.* 221, 470–477). These results link for the first time endogenous mono(ADP-ribosylation) to Ca^{2+} signaling, and support previous findings indicating that PLC-beta may be recruited as part of the apoptotic signaling.

Expression of a protein repair methyltransferase prevents apoptosis induced by oxidative stress in endothelial cells

Amelia Cimmino[1,2], **Diego Ingrosso**[1,2], **Lucia Masella**[1], **Rosanna Capasso**[1], **Vincenzo Zappia**[1,2], and **Patrizia Galletti**[1,2]

[1] Department of Biochemistry and Biophysics ''F. Cedrangolo'', School of Medicine and Surgery, Second University of Naples, via Costantinopoli 16, 80138 Naples, Italy

[2] Cardiovascular Research Center, School of Medicine and Surgery, Second University of Naples, via De Crecchio 7, 80138 Naples, Italy

Background. Protein deamidation/isomerization at asparaginyl/aspartyl sites is a spontaneus post-biosynthetic protein modification leading to the formation of racemized and/or isomerized aspartyl residues. These particularly occur during cellular and molecular aging, and are also increased under physical and chemical cell stress conditions. L-isoaspartyl residues are recognized and modified by protein L-isoaspartyl *O*-methyltransferase (PIMT; EC 2.1.1.77) a S-adenosylmethionine-dependent enzyme, which is involved in the repair of such altered residues. It has also been shown that PIMT is inhibited in hyperhomocysteinemia, a condition associated with high cardiovascular risk.

Aim of this work was the evaluation of the biological role of PIMT in the genesis of endothelial cell damage and the definition of its possible role in the apoptosis induced by oxidative stress.

Methods. Porcine endothelial cells (PAE) were transfected with pcDNA3.1 plasmid constructs containing the wild type or the antisense or mutants of human PIMT cDNA. Cell stress was induced by H_2O_2 treatment. Apoptosis was detected by monitoring DNA fragmentation, as well as caspase 3 and PARP activation.

Results. PAE cells, transfected with a plasmid containing the wild type *PIMT* gene, overexpress the repair methyltransferase, compared to controls. Transfection with antisense *PIMT* gene blocks expression of the endogenous methyltransferase. Transfection with plasmid constructs, containing various *PIMT* site-directed mutations of a highly conserved residue (Asp_{83}) located in the active site of the enzyme, blocks endogenous PIMT activity. Treatment with 0.1 mM H_2O_2 induces apoptosis in PAE cells in which PIMT expression or activity are inhibited. This concentration threshold is significantly increased (0.4 mM H_2O_2) in cells transfected with wild type *PIMT* gene. Therefore, PAE transfected with antisense or mutant *PIMT* gene are particularly sensitive to apoptosis induced by oxidants, even at the lowest H_2O_2 concentrations employed. Moreover, blockade of methyltrasferase expression or function, by trasfection with antisense or mutant *PIMT* gene, is associated with the accumulation of deamidated/isomerized damaged proteins, upon oxidative stress. The protective effect of PIMT towards apoptosis induced by oxidants is specifically due to its enzymatic activity. In fact transfection with mutants bearing a site-directed mutations within the catalytic site of the enzyme specifically abolishes this effect.

Discussion and conclusion. PIMT activity is able to prevent apoptosis, induced by oxidative stress in cell cultures through activation of caspase3/PARP common pathway. Such a protective effect is linked to the methylation/repair activity of this enzyme toward deamidated/isomerized proteins, triggered by oxidative stress generated by H_2O_2 cell treatment.

Post-translational modifications in the Swiss-Prot protein knowledgebase

Nathalie Farriol-Mathis and **Amos Bairoch**

Swiss Institute of Bioinformatics, Geneva, Switzerland

Proteins are subject to three classes of protein modifications: pre-, co- and post-translational modifications. Most of them are made when the protein is already folded, otherwise known as post-translational modifications (PTMs). Some are made co-translationally, while the polypeptide is still being synthesized on the ribosome. A few 'non-standard' amino acids (e.g. selenocysteine, pyrrolysine) are incorporated into proteins following the modification of 'standard' amino acids as they are charged on tRNAs; these are pre-translational modifications.

PTMs are involved in many processes such as protein turnover, protein-protein interactions, subcellular targeting and signal transduction.

The 20 amino-acid basic set is significantly increased to nearly 300 by various PTMs, thus leading to a dramatic expansion in protein diversity. In Swiss-Prot, we have been providing our users with experimental PTM data for a long time and we strive to increase the number and variety of PTM records continuously.

Experimental data is just pouring in thanks to the field of proteomics where mass spectrometry combined with protein separation methods allow large-scale analysis of tissular extracts and the subsequent identification of modified proteins as well as the sites of modification.

Predicted data, provided by in-house evaluated bioinformatics tools (e.g. N-linked glycosylation, myristoylation, sulfation, addition of GPI-anchor, signal processing predictors), are carefully annotated and bear a special tag. More PTM prediction tools are developed inside and outside of the SIB.

Recently, links to specific PTM-oriented databases such as 'GlycoSuiteDB' and 'Phosphorylation Site Database' were integrated, thus illustrating our effort to connect biological information within the various databases.

Insertion of sulfonyl analogues of β^3-amino acids in peptides. Synthesis and activity of Gonadotropin-Releasing Hormone analogues

C. Giordano[1], **S. Di Segni**[1], **Y. Koch**[2], **S. Rahimipour**[2], **N. Ben-Aroia**[2], and **M. Fridkin**[2]

[1] Istituto di Chimica Biomolecolare (CNR), Sezione di Roma, c/o Dipartimento di Studi Farmaceutici, Università ''La Sapienza'', P.le A. Moro 5, 00137 Rome, Italy

[2] Department of Organic Chemistry and Neurobiology, Weizmann Institute of Science, Rehovot 76100, Israel

The design, synthesis and biological activity of a new class of Gonadotropin-Releasing Hormone (GnRH) analogues is described. In these compounds the native Gly^6-Leu^7 amide bond, involved into the endopeptidases cleaving processes, is replaced by the $-CH_2SO_2NH-$ junction. Four GnRH analogues containing the -$Xaa^6\Psi(CH_2SO_2NH)Leu^7$-sequence [with Xaa = Gly (**1**), Ala (**2**), Val (**3**) and Phe (**4**)] were prepared, where the 2-aminoethanesulfonyl residue maintains the characteristic side chain and the chirality (compounds **2**–**4**) of the corresponding proteinogenic aminoacid. The β-alanine incorporating analogue [β-Ala^6]GnRH (**5**) was also synthesized in order to compare its activity with that of **1**, from which it differs for the presence of a CO–NH in place of a SO_2–NH junction. Finally, a pseudo-nonapeptide (**6**), representing a reduced-size analogue of **4,** in which the Tyr^5 is not present, was also prepared and tested. Enzymatic degradation tests showed an increased stability, in comparison with that of the native hormone, for all the β-sulfonamido GnRH analogues and for the β-Ala-containing peptide. Biological activity, based on LH release from dispersed pituitary cells by the GnRH analogues, was very similar to that of the native hormone for the β-Ala-containing peptide, but seemed to be lower for all the β-sulfonamido analogues.

Role of elongation factor 1A (EF-1A) and ubiquitin in INFa and VP16-mediated apoptosis

O. Longo[1], **M. Marra**[2], **A. Lamberti**[1], **M. Caraglia**[2], **A. Abbruzzese**[2], and **P. Arcari**[1]

[1] Department of Biochemistry and Medical Biotechnology, University of Naples Federico II, and
[2] Department of Biochemistry and Biophysic, II University of Naples, Italy

An increasing series of data are presently emerging concerning the involvement of translation factors in control of cell proliferation, thus suggesting that protein synthesis can be an additional target for anti-cancer strategies. Among these, wide evidence suggests the involvement of the elongation factors 1A (EF-1A) at the onset of oncogenesis. Altered expression of translation elongation factor, a core component of protein synthesis, and closely related sequences have been linked to transformed phenotypes in several independent studies and in diverse systems. To understand the functional role of the elongation factor 1A in the regulation of cell proliferation and apoptosis a human epidermoid lung cancer cell line (H1355) was used. Apoptosis was induced by treatment of the cells with interferon α (INFα) or VP16. Treatment with alpha interferon (1000 IU/ml) leads not only to inhibition of cell proliferation and apoptosis, but also to an increase of the epidermal growth factor receptor (EGF-R) function and expression. We firstly analysed both EF-1A mRNA and EF-1A protein levels in INFα or VP16 treated and not treated cells by Northern and Western blots. The results obtained showed that there was a reduction in the levels of the protein at 5–10 minutes treatment with INFα. After 20 minutes the EF-1A levels went back to the level of the control. In the case of VP16, the reduction of EF-1A levels was instead observed after 20 and 30 minutes treatment with a recovery that appeared after 180 minutes. The EF-1A mRNA levels appeared not affected by the treatment of the cells with of both INFα and VP16. Because type I INFs induce a 15 kDa protein similar to ubiquitin, we have investigated whether IFNα may alter the intracellular stability of EF-1A via modulation of its ubiquitination. For this purpose, IFNα or VP16 treated cells extracts were immunoprecipitated with anti-EF-1A monoclonal antiboby and then assayed by Western blot using anti-ubiquitin polyclonal antibody. The results indicated that EF-1A is involved in the process of protein degradation mediated by ubiquitin.

Melatonin reduces stress-induced apoptosis in U937 cells by stimulating mt1 plasma membrane receptors

L. Paternoster[1], **C. Cerella**[1], **M. D'Alessio**[1], **M. De Nicola**[1], **G. Clavarino**[1], **C. Albertini**[3], **A. Accorsi**[3], **G. Spadoni**[4], **G. Tarzia**[4], **A. Bergamaschi**[2], and **L. Ghibelli**[1]

[1] Dipartimento di Biologia,
[2] Cattedra Medicina del Lavoro, Università di Roma Tor Vergata,
[3] Istituto Chimica Biologica, and
[4] Istituto Chimica Farmaceutica, Università di Urbino, Italy

Melatonin, the main product of the pineal gland, is involved in many light-related processes. Melatonin is synthesized from Trypthophane, which is converted in serotonin, and then melatonin, in a two-step pathway involving N-acetylation and O-methylation. The cellular effects of melatonin are mediated by plasma membrane high-affinity receptors, namely Mel1a (mt1), Mel1b (mt2), Mel1c (mt3), that are coupled to G proteins, thus initiating a signal transduction pathway. Melatonin, being a lipophilic compound, freely diffuses through biological membranes, and can be internalised into cells. Melatonin is also known to exert an antioxidant activity; thus, the cellular effects may either be receptor-mediated, or occur via radical scavenging.

With the aim of analysing the effects of melatonin on apoptosis, and investigating the mechanisms involved (antioxidant activity vs. receptor stimulation), we probed melatonin on two human tumor cells (monocytic U937 and EBV+ lymphoma E2R), treated with different apoptogenic agents, using as tools a pair of melatonin analogues with increased or decreased receptor affinity but maintaining the radical scavenger ability.

We found that in U937 cells (expressing the mt1 receptor), melatonin and related compounds protect from apoptosis to an extent that is directly proportional to their ability to stimulate the receptors (evaluated as the extent of cytosolic Ca2+ fluxes raised by each melatonin related compound). Instead, in E2R cells (probably devoid of receptors) melatonin is not able to prevent apoptosis.

Our data demonstrate that the effects of melatonin on apoptosis are mediated by receptor stimulation and not necessarily involve the radical scavenging.

Unbalanced methylation of macromolecules: a new mechanism for homocysteine toxicity on cardiovascular system?

Diego Ingrosso[1,3], **Amelia Cimmino**[1,3], **Alessandra F. Perna**[2,3], **Lucia Masella**[1,3], **Natale G. De Santo**[2,3], **Maria Luigia De Bonis**[4], **Marcella Vacca**[4], **Maurizio D'Esposito**[4], **Michele D'Urso**[4], **Patrizia Galletti**[1,3], and **Vincenzo Zappia**[1,3]

[1] Department of Biochemistry and Biophysics "F. Cedrangolo",
[2] First Division of Nephrology/Department of Pediatrics,
[3] Cardiovascular Research Center, School of Medicine and Surgery, Second University of Naples, and
[4] Institute of Genetics and Biophysics "A. Buzzati Traverso", CNR, Naples, Italy

Homocysteine (Hcy) is a sulfur amino acid whose increase in plasma concentration (hyperhomocysteinemia) is a strong and independent cardiovascular risk factor for myocardial infarction, stroke and peripheral arterial and venous thrombosis. Hyperhomocysteinemia is associated with a number of genetically determined and acquired conditions, and is highly prevalent in patients with uremia, a population characterized by a high mortality rate for cardiovascular disease. While the epidemiological link between high homocysteine (Hcy) and cardiovascular disease is well established, the underlying molecular mechanism(s) is still poorly understood. A major biochemical alteration occurring during hyperhomocysteinemia is the increase of S-adenosylhomocysteine (AdoHcy): the *in vivo* Hcy precursor and a powerful competitive inhibitor of S-adenosylmethionine (AdoMet)-dependent methyltransferases. Previous data showed that hyperhomocysteinemia is accompanied by: A) a significant reduction of [AdoMet]/[AdoHcy] ratio, both intracellularly and in plasma. B) Inhibition of a methyltransferase involved in the repair of aged/damaged proteins (PIMT; EC 2.1.1.77). C) These alterations can be at least partially corrected by folate administration.

Evidence for both S-adenosylhomocysteine accumulation and derangement of protein methylation/repair reaction in hyperhomocysteinemia led us to hypothesize that several other methyl transfer reactions could be variously affected, bringing forth the concept of "unbalanced methylation" status.

We recently found, in uremic/hyperhomocysteinemic patients: i) a decrease of total DNA methylation levels, which correlates with plasma homocysteine concentrations; ii) an alteration of allelic expression in both sex-linked and imprinted genes, which are known to be regulated by methylation; the shift from monoallelic to biallelic expression depends on the degree of hyperhomocysteinemia; iii) folic acid therapy is able to restore DNA methylation levels to normal and correct altered gene expression patterns. These data provide a description of a metabolic alteration, occurring in an acquired disease, which affects the epigenetic control of gene expression, that can be reverted by vitamin treatment.

Results as a whole demonstrate that hypomethylation of macromolecules (proteins and DNA) is a major biochemical alteration in hyperho-

mocysteinemia, thus supporting the ''unbalanced methylation'' hypothesis, as a mechanistic model for Hcy toxicity on cardiovascular system.

Protein prenylation as a potential target in the therapy of human neoplasms

Michele Caraglia, Anna Maria D'alessandro, Monica Marra, Gaia Giuberti, Giuseppe Bossa, Lucia Lubrano Lavadera, Giovanni Martin, and **Alberto Abbruzzese**

Dipartimento di Biochimica e Biofisica, Seconda Università di Napoli, Italy

Post-translational modification of proteins by the addition of a farnesyl or a geranyl-geranyl group is critical for the function of a number of proteins involved in signal transduction. Prenylation facilitates their membrane association and also promotes protein-protein interaction. Recently, progress has been made in understanding the biological significance of prenylation. First, effects of farnesyltransferase inhibitors (FTIs) on cancer cells have been examined using a variety of human cancer cells. This study showed that one of the major effects of FTIs is to alter cell cycle progression. Both G0/G1 enrichment and G2/M accumulation were observed depending on the cell line examined. Many physiological and oncogenic activities of the ''classical'' Ras proteins (H-Ras, N-Ras and K-Ras4A and -4B, Rho, Rac etc.) require their correct localization to the plasma membrane. Geranyl-geranyl transferase inhibitors have been also designed and studied. Moreover, bisphosphonates (BPs) are an emerging class of drugs with the most important place in the palliative care of cancer patients for the treatment of hypercalcaemia and skeletal complications. Recent reports suggest that these drugs may also directly act on cancer cells, affect the isoprenylation of intracellular proteins involved in signal transduction such as small G proteins, inhibiting farnesyl-pirophosphate synthase, and induce apoptosis in several tumour cells. We have demonstrated that the most potent anti-resorptive and nitrogen-containing BP Zoledronic acid (ZOL) induces apoptosis and cell growth inhibition dependent from ras- > erk and ras- > akt pathway inactivation and caspase 9 stimulation in human pancreatic cancer cells. Similar data were also obtained in human epidermoid cancer cells. Moreover, the combination of ZOL with the FTI R115777 (Zarnestra) was synergistic on the apoptosis and growth inhibition of these cells. The integrated use of agents that inhibits protein prenylation at different biochemical levels can be useful in the design of new anticancer strategies.

Neurobiology

Effect of chronic glutamate administration to pregnant rats during gestation on metabotropic glutamate receptors from mothers and full-term fetuses brain

David León, Inmaculada Iglesias, María Ángeles Ruiz, José Luis Albasanz, and **Mairena Martín**

Departamento de Química Inorgánica, Orgánica y Bioquímica, Centro Regional de Investigaciones Biomédicas, Universidad de Castilla-La Mancha, Spain

Glutamate is the main excitatory neurotransmitter in the Central Nervous System that is involved in physiological functions, such as learning and memory. These functions are mediated through specific receptors which have been classified into ionotropic and metabotropic receptors. Metabotropic glutamate receptors (mGluR) are coupled, through G proteins, to phospholipase C stimulation or adenylyl cyclase inhibition. Like other G protein coupled receptors, mGluR are regulated by agonist exposure. The aim of this work was to study the effect of chronic L-Glutamate intake during pregnancy on mGluR/phospholipase C (PLC) transduction pathway in membranes from both mothers and fetuses rat brain. L-Glutamate treatment caused a significant decrease in total mGluR binding detected in mothers brain, while no changes were observed in fetal brain membranes. Levels of mGluR1, αGq/11 and PLCβ1 proteins, detected by immunoblotting assays, were significantly decreased in mothers brain, however, no variation in the level of these proteins was observed in fetuses brain. Moreover, the decrease of different components of mGluR transduction pathway in maternal brain was associated with a significant loss of receptor functionality, determined by the stimulatory ability of mGlu receptor agonists on PLC activity. These results suggest that chronic L-Glutamate intake during gestation desensitizes mGluR/PLC transduction pathway in mothers without significant alterations in full-term fetuses brain. The absence of variation on mRNA coding mGluR/PLC system components could involve intracellular organelles participation in this maternal brain regulation.

Glutathione transferase of patients with Parkinson's disease

T. Ferraro, A. Bagalà, L. Bastone, N. Romeo, P. Serra, and **L. Crescibene**

Biochemisty Laboratory, Institute of Neurological Sciences-CNR-Mangone, Cosenza, Italy

Parkinson's Disease (PD) is a slowly progressing disease of the nervous system. The loss of dopaminergic neurons in PD results in enhanced metabolism of dopamine, augmenting the formation of H2O2, thus leading to generation of highly neurotoxic hydroxyl radicals (OH′). The most important free radical scavenger in the cells of the substantia nigra is the powerful brain antioxidant, glutathione. Studies have shown that age-related loss of glutathione in the dopaminergic neurons of the substantia nigra can bring about various changes in the cells. These changes, in combination with dopamine oxidation, appear to hasten cell death and advance the progression of PD.

Glutathione transferases (GSTs) are an ubiquitous group of detoxification enzymes involved in the metabolism of pesticides and other toxins. GSTs have direct antioxidant activity and are involved in the metabolism of dopamine. GSTs are catalyst of reactions in which reduced glutathione (GSH) acts as a nucleophile, conjugating to and facilitating removal or reduction of the second substrate. We have studied the levels of GST enzyme activity in leucocytes of 45 patients with PD and a control group of 40 subjects, compared by age and sex. GST activity was determined using 1-chloro-2,4-dinitrobenzene as substrate.

Protein concentrations were measured by the method of Lowry.

Significant differences ($p < 0.001$) of enzyme activity were found in leucocytes between patients with PD (3.79 nmoli/mg protein/min $\pm$ 1.08) and controls (6.89 nmoli/mg protein/min $\pm$ 1.66).

The results of this study support the possible involvement of oxidative stress in the pathogenesis of Parkinson's disease.

Myelin basic protein and Multiple Sclerosis

T. Ferraro, A. Bagalà, L. Bastone, N. Romeo, G. Di Palma, and **L. Crescibene**

Biochemisty Laboratory, Institute of Neurological Sciences-CNR-Mangone, Cosenza, Italy

Multiple Sclerosis (MS) causes localised demyelination in the central nervous system. Myelin basic protein (MBP) is the major protein component of myelin, and the concentration of MBP or its fragment in cerebrospinal fluid (CSF) correlates closely with the clinical activity of MS and other diseases in which there is myelin breakdown.

We used a sensitive and specific ELISA method for MBP and measured MBP in CSF from 23 patients with MS and 23 other neurological diseases. All CSF samples were obtained by lumbar puncture and were stored at -80°C until assayed.

The ELISA is an enzymatically amplified two-step sandwich-type immunoassay. In the assay, standards, controls and CSF samples are incubated in microtritation wells, which have been coated with anti-human MBP antibody. After incubation and washing, the wells are treated with another anti-human MBP detection antibody labeled with the enzyme horseradish peroxidase. After a second incubation and washing step, the wells are incubated with the substrate tetramethylbenzidine (TMB). An acid stopping solution is then added and the degree of enzymatic turnover of the substrate is determined by dual wavelength absorbance measurement at 450 and 620 nm. The absorbance measured is directly proportional to the concentration of MBP present. The mean CSF MBP concentration in the patients with other neurological diseases was 0,34 ng/ml. The mean CSF MBP concentration in the MS patients was 0,38 ng/ml. Our results show that the CSF levels of MBP were not different in MS patients than in other neurological diseases.

Imaging by two photon laser scanning microscopy of calcium accumulations in neurites and cell bodies of rat cerebellar granule cells in culture

A. Cupello[1], **A. Esposito**[2], **C. Marchetti**[3], **F. Pellistri**[2], and **M. Robello**[2]

[1] IBFM, CNR, Sezione di Genova, Italy
[2] INFM, Università di Genova, Italy
[3] Istituto di Biofisica, CNR, Sezione di Genova, Italy

The specific subcellular compartment involved in intracellular accumulation of Ca^{2+} is of para mount importance in determining the ultimate biochemical and physiological consequences of increases of intracellular calcium concentrations. Taking into account this circumstance, we studied topical accumulations of calcium ions in neurites and cell bodies by two-photon laser scanning microscopy, in cerebellar granule cells in culture loaded with the fluorescent indicator Oregon Green 488 Bapta. High potassium had a different effect on calcium accumulation in cell bodies and neurites. In cell bodies there was a peak accumulation of calcium ions which was then followed by a steady state above the basal level, in neurites the peak accumulation was followed instead by a return to the basal level. When neurons were stimulated by NMDA, intracellular calcium reached a steady state level and remained constant until the agonist was in the bath both in the cell bodies and neurites.

The implications of these results on the functional coupling between $GABA_A$ and glutamate NMDA receptors are discussed.

$GABA_A$ receptor subunits in rat cerebellum granule cells in culture

A. Cupello[1], **C. Luccardini**[1], **F. Merlo**[2], and **M. Robello**[2]

[1] IBFM, CNR, Sezione di Genova, Italy
[2] INFM, Università di Genova, Italy

The main subunits expressed in cerebellar granules $GABA_A$ receptors (α_1, α_6, $\beta_{2/3}$, γ_2, δ) have been studied by immunocytochemistry and the two-photon microscopy technique. The various subunits present specific distributions in granule cells obtained from neonatal rats and kept in culture. The most interesting findings appear to be the following. First, whereas the α_6 subunit is present on almost every neurite expressed by the granules, the γ_2 subunit appears only in neurites which seem to have a dendritic nature. Second, both α_6 and γ_2 subunits appear to accumulate in the cytoplasmic domains immediately below the cone of emergence of neurites. This suggests a conspicuous transport of such subunits from the site of synthesis in the cell body to the site of final expression in the neurites (dendrites and/or axon terminals). Finally, we show that the distribution of the plasma membrane ubiquitous $\beta_{2/3}$ subunits is not altered by disruption of either actin microfilaments or microtubules. However, their distribution is heavily affected by disruption of both cytoskeletal structures.

D-Aspartic acid in the nervous system of *Aplysia*: role in learning and memory

A. D'Aniello[1], **P. Spinelli**[1], **A. De Simone**[1], **S. D'Aniello**[1], **J. Rios**[2], **K. Peterson**[2], **M. Tsesarskaja**[2], and **G. Fisher**[2]

[1] Laboratory of Neurobiology, Stazione Zoologica, 80121 Napoli, Italy
[2] Department of Chemistry, Barry University, Miami Shores, FL 33161, USA

D-Aspartic acid (D-Asp) is an endogenous amino acid found in the nervous tissues of various animals. Here, we show that in the nervous ganglia of two species of *Aplysia* (*Aplysia californica* and *Aplysia limacina*) D-Asp is present in high concentration. Analysis carried out on various isolated neurons, indicated that the sensitive neurons are the type of neuron richest of D-Asp. Furthermore, we observe that if the animals are subjected to learning and memory experiments, by using water pick stimulation or by electric stimulation, the concentration of D-Asp significantly increases above all in the sensitive neurons compared to the controls suggesting that D-Asp is involved in learning and memory processes. Aspartate racemase, the enzyme that transforms L-Asp into D-Asp is present in the same tissues and also increases significantly in the trained animals. Immunohistochemical studies conducted on the entire ganglia of *Aplysia* from control animals and from trained animals indicated that D-Asp is localized in both cytoplasm and nucleus of the neurons. However, in the trained animal it increase significantly in the nucleus suggesting a possible role of D-Asp in the promote the synthesis of proteins implicated in the learning and memory process.

The blood-brain barrier: dysfunction and recovery

A. G. de Boer and **P. J. Gaillard**

Blood-Brain Barrier Research Group, Department of Pharmacology, Leiden-Amsterdam Center for Drug Research, University of Leiden, 2300 RA Leiden, The Netherlands

Background: The blood-brain barrier (BBB) is the interface between blood and brain (1). In this respect it protects and controls the homeostasis of the brain. Various diseases with an inflammatory component such as ischemic stroke, head trauma, multiple sclerosis, meningitis, etc., disturb the integrity of the BBB and therefore the homeostasis of the brain.

Hypothesis: Therefore, we wanted to test the influence of inflammatory mediators with respect to BBB dysfunction and the protection of BBB integrity by anti-inflammatory compounds.

Methods: The effects of inflammatory stimuli were studied with an *in vitro* model of the BBB (2). Dysfunction and protection was investigated by measurement of the transport of the hydrophilic marker compound fluorescein and the trans-endothelial-electrical resistance (TEER).

Results: We found that compounds like lipopolisaccharide (LPS) and nitrogen monoxide (NO) increased the paracellular permeability of the BBB. These effects could be prevented by pretreatment with the radical scavenger N-acetylcysteine. In addition, compounds like glucocorticoids and interferon-α, β closed the BBB and decreased its paracellular permeability.

Conclusion: Our results indicate that inflammatory stimuli open the *in vitro* BBB and that radical scavengers are able to protect the BBB against these stimuli. Similarly glucocorticoids and interferon-α, β can be used to restore the barrier properties of the *in vitro* BBB.

Distinction between the neurotransmitter functions of glutamate and aspartate revealed by *in vivo* microdialysis

L. Ballini, L. Bianchi, M. Fattori, M. A. Colivicchi, C. Mucignat-Caretta, A. Cavaggioni, K. F. Tipton, M. G. Giovannini, G. Pepeu, and **L. Della Corte**

Dipartimento di Farmacologia Preclinica e Clinica "M. Aiazzi Mancini", Universitá di Firenze, Viale G. Pieraccini 6, 50139 Firenze, Italy

It has proven difficult to distinguish separate functions for L-glutamate and L-aspartate as neurotransmitters. This is because aspartate may be taken up by the presynaptic glutamate transporter, stored in glutamate vesicles released on stimulation of glutamatergic neurones and act as an agonist of some glutamate receptors. This report describes the results of studies, using *in vivo* microdialysis in the freely removing rat, that show a clear distinction between evoked release of glutamate and aspartate in two very different model systems and in two different brain regions. Amino acid release was determined by hplc, with fluorimetric detection of their *o*-phthalaldehyde derivatives. In the first of these studies exposure of oestrus female rats to urinary pheromones was shown to induce an immediate behavioural activation together with release of aspartate and glutamate in the posteromedial cortical nucleus of the amigdala. However, aspartate but not glutamate was associated to the second delayed peak of behavioural activation. In the second and unrelated system studied, exploration of a novel environment was shown to result in the release of aspartate and, to a lesser extent, glutamate in the ventral hippocampus. A second exposure to the same environment induced the release of aspartate but not glutamate. In both studies administration of tetrodotoxin (3 μM) inhibited the aspartate release, consistent with it originating from nerve terminals. These data demonstrate the power of microdialysis in providing the evidence for a distinct role of aspartate from that of glutamate as neurotransmitters.

Molecular mechanisms involved in T cell migration across the blood-brain barrier

Britta Engelhardt

Max-Planck Institute of Vascular Biology, Münster, Germany

In the healthy individuum lymphocyte traffic into the central nervous system (CNS) is very low and tightly controlled by the highly specialized blood-brain barrier (BBB). In contrast, under inflammatory conditions of the CNS such as in multiple sclerosis or in its animal model experimental autoimmune encephalomyelitis (EAE) circulating lymphocytes and monocytes/macrophages readily cross the BBB and gain access to the CNS leading to edema, inflammation and demyelination. Interaction of circulating leukocytes with the endothelium of the blood-spinal cord and blood-brain barrier therefore is a critical step in the pathogenesis of inflammatory diseases of the CNS. Leukocyte/endothelial interactions are mediated by adhesion molecules and chemokines and their respective chemokine receptors. We have developed a novel spinal cord window preparation, which enables us to directly visualize CNS white matter microcirculation by intravital fluorescence videomicroscopy. Applying this technique of intravital fluorescence microscopy we could provide direct *in vivo* evidence that encephalitogenic T cell blasts interact with the spinal cord white matter microvasculature without rolling and that -integrin mediates the G-protein independent capture and subsequently the G-protein dependent adhesion strengthening of T cell blasts to microvascular VCAM-1. LFA-1 was found to neither mediate the G-protein independent capture nor the G-protein dependent initial adhesion strengthening of encephalitogenic T cell blasts within spinal cord microvessel, but was rather involved in T extravasation across the vascular wall into the spinal cord parenchyme. Our observation that G-protein mediated signalling is required to promote adhesion strengthening of encephalitogenic T cells on BBB endothelium *in vivo* suggested the involvement of chemokines in this process. We found functional expression of the lymphoid chemokines CCL19/ELC and CCL21/SLC in CNS venules surrounded by inflammatory cells in brain and spinal cord sections of mice afflicted with EAE suggesting that the lymphoid chemokines CCL19 and CCL21 besides regulating lymphocyte homing to secondary lymphoid tissue are involved in T lymphocyte migration into the immuneprivileged CNS during immunosurveillance and chronic inflammation. In my presentation I will summarize our current knowledge on the sequence of traffic signals involved in T lymphocyte recruitment across the healthy and inflamed blood-brain and blood-spinal cord barrier based on our *in vitro* and *in vivo* investigations.

Analysis of *in vivo* imaging of angio- and retinopathy in correlation with an expression of potential molecular markers for high risk of secondary complications in patients with Diabetes mellitus

Olga Golubnitschaja[1], A. Jaksche[2], Stefanie Karl[2], Heike Mönkemann[1], Kristina Yeghiazaryan[1], Hans H. Schild[1], and **Karin U. Löffler[2]**

[1] Department of Radiology and
[2] Department of Ophthalmology, University of Bonn, Sigmund-Freud-Str. 25, D-53105 Bonn, Germany

Objective: As a consequence of hyperglycemia the deposition of advanced glycation end products is enhanced in *Diabetes mellitus* (DM) and has been linked to diabetic complications such as microvascular disorders. Glycated proteins have receptors on mononuclear blood cells (MBCs) and have been shown to generate reactive oxygen species (ROS) altering gene expression and modifying cellular targets, such as endothelial cells. Both angio- and retinopathy are secondary complications observed in DM-patients. The retinal degeneration in DM might be linked to both the retinal neovascularization and damaging effect of generated ROS, since inflammatory cells can infiltrate retina. Because of the central role of activated MBCs in the regulation of target cells, we hypothesized a functional link between specific changes in gene expression of MBCs, activity of extracellular matrix (ECM) remodelling proteins, and extent of retinal angiopathy in DM-patients.

Results and conclusions: On the level of transcription an expression of 84 genes varied in DM-group and differed from that of controls. 7 proteins were detected to be expressed only in the DM-group while 15 other proteins were lacking there. Differential gene expression was found further between female and male DM-subgroups, and the activity of ECM degrading metalloproteinase-9 was more pronounced in serum of male DM-patients. We concluded that the frequency and severity of secondary DM-complications might be gender-dependent. We proposed some potential molecular markers, such as metalloproteinases, recoverin and retinoblastoma-associated protein 1, to be considered for *ex vivo* monitoring and *in vivo* imaging to estimate the risk of angio- and retinopathy in DM.

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Effects of metabolic insults and perinatal asphyxia on CNS plasticity

M. Herrera-Marschitz[1,2], D. Bustamante[1], P. Morales[1], V. Klawitter[1], M. Cordero[1], P. Huaiquín[1], M. L. Bustamante[1], and **M. Goiny[2]**

[1] Programme of Molecular & Clinical Pharmacology, ICBM, Medical Faculty, University of Chile, Santiago, Chile
[2] Department of Physiol. & Pharmacol., Karolinska Institutet, Stockholm, Sweden

The paper refers to a research line aiming to study the plasticity of the central nervous system (CNS) following perinatal asphyxia. The

hypothesis is that perinatal asphyxia primes the development of the neurotransmission systems in the CNS.

The issue is investigated using an experimental model of perinatal asphyxia in rats, consisting in immersing into a water bath foetuses-containing uterus horns removed from ready-to-deliver rats for different periods of time. After asphyxia, surrogate dams nurse the pups until further *in vitro* or *in vivo* experiments. Shortly after delivery, the pups are used for preparing organotypic cultures, and grown for one month *in vitro*, measuring cell survival, neurocircuitry development and markers for neurogenesis, with morphological and neurochemical methods. When preserved alive for long periods, the animals are studied *in vivo* with microdialysis or killed for *ex vivo* tissue biochemistry.

We have found that perinatal asphyxia alters neuronal connectivity, mainly that of dopamine systems, affecting neuritogenesis, dopamine synthesis, dopamine release and the regional expression of dopamine receptors. Furthermore, there is an effect on neurogenesis, labelled with BrdU in hippocampus, which is significantly increased one week after the perinatal insult.

The goal is to identify vulnerable parameters and strategies against the long-term consequences of anoxic insults early in life. Thus, we have found and proposed that replacing NAD + and ATP levels with nicotinamide can represent a novel therapeutic strategy, even when the treatment is started 24 h after a perinatal asphyctic insult.

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Combined analysis of biochemical parameters in serum and potential molecular markers in circulating leukocytes as an *ex vivo* monitoring system to estimate a risk of developing complications in Diabetes mellitus

Marko Kapalla[1], **Kristina Yeghiazaryan**[2], **Heike Mönkemann**[2], **Marta Hricová**[1], **Antonín Pírek**[1], **Hans H. Schild**[2], and **Olga Golubnitschaja**[2]

[1] Department of Clinical Biochemistry and Haematology, Alpha medical s.r.o., Podhora 47, Ružomberok, Slovakia

[2] Department of Radiology, University of Bonn, Sigmund-Freud-Str. 25, Bonn, Germany

Objective: Oxidative stress plays a crucial role in the development of diabetic complications. Individual sensitivity against the stress, however, varies among diabetic patients and results, therefore, in differential severity of consequent complications such as angio-, cardio- and neuropathy. To allow more complex interpretation of a delicate antioxidant/ free radicals balance and its effect on cellular functions in an individual diabetic patients, we analysed correlations between total antioxidant status (TAS), antioxidant gap (AtxGap), level of free radicals (FR), routine clinical biochemical parameters in blood and differential gene expression in circulating leukocytes of diabetic patients vs. non-diabetic individuals.

Results and conclusions: Positive correlation was found between TAS and creatinine ($p = 0.05$), AtxGap and iron ($p = 0.025$), and between AtxGap and anti-streptolysin O ($p = 0.025$). Whereas no correlation was found between FR and any of the routine clinical parameters tested, a negative correlation was observed between AtxGap and glucose content ($p = 0.025$). A decrease in antioxidant status, judged by determined TAS, AtxGap and FR, significantly influenced expression of selected stress responsible genes in leukocytes: whereas the level of NF-kappaB, XRCC1 and 90-kDa heat-shock protein A transcript rates was higher in all diabetic patients compared to controls, an expression of XIAP, cytochrome P450 reductase, ATP-dependent DNA ligase I and microsomal stress 70 protein ATPase core was up-regulated only in patients with the decreased TAS level. The differential expression of the latter group of genes might be further considered as potential marker for an increased risk of diverse diabetic complications helping also in reliable monitoring of supplemental antioxidant therapy.

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L-Dihydroxyphenylalanine treatment reduces hydroxyl radical levels in tissue and in *in vivo* microdialysates of dopamine-denervated rat striatum

R. M. Kostrzewa[1], **J. P. Kostrzewa**[1], **R. Brus**[2], and **P. Nowak**[2]

[1] Department of Pharmacology, Quillen College of Medicine, East Tennessee State Univ., Johnson City, TN 37614, USA

[2] Department of Pharmacology, Medical Univ. of Silesia, 41-808 Zabrze, Poland

The hypothesized role of L-dihydroxyphenylalanine in generating hydroxyl radical in Parkinsonians was addressed by first producing an animal model of Parkinson's disease, treating acutely with L-dihydroxyphenylalanine, and assessing hydroxyl radical in dopamine-denervated striatal tissue and its *in vivo* microdialysate. The neurotoxin 6-hydroxydopamine (134 mcg) was injected into the lateral ventricles of rats at 3 days after birth to destroy nigrostriatal dopaminergic nerves (i.e., 99% depletion of endogenous dopamine of striatum). In these rats as adults, acute L-dihydroxyphenylalanine (60 mg/kg; carbidopa pretreatment) partly restored DA levels in striatal tissue and its *in vivo* microdialysate. Simultaneously, as indicated by the salicylate spin trap product 2,3-dihydroxybenzoic acid, hydroxyl radical levels were reduced in dopamine-denervated striatal tissue and its microdialysate by L-dihydroxyphenylalanine treatment. These findings indicate that L-dihydroxyphenylalanine is beneficial not only in restoring dopamine but also in reducing hydroxyl radicals in a Parkinsonian striatum. (Supported by NS 39272 and NN-2-007/03.)

Modulation of ventral pallidal dopamine and glutamate release by the intravenous anesthetic propofol studied by *in vivo* microdialysis

C. Grasshoff[1], **M. Herrera-Marschitz**[3,4], **M. Goiny**[3], and **B. D. Kretschmer**[2]

[1] Department of Anesthesiology and Transfusion Medicine, University of Tübingen, and

[2] Department of Neuropharmacology, University of Tübingen, Tübingen, Germany

[3] Department of Physiology and Pharmacology, Karolinska Institute, Stockholm, Sweden

[4] Programme of Molecular & Clinical Pharmacology, ICBM, Medical Faculty, University of Chile, Chile

The intravenous anesthetic propofol is reported to have various psychological side effects as hallucinations, sexual disinhibition, or euphoria. Hedonic and rewarding states like these are modulated by the dopaminergic system in the nucleus accumbens, prefrontal cortex and also in the ventral pallidum and by the glutamatergi system in the neocortex and limbic system. In the present study, propofol was administered either alone or in combination with the GABAA receptor antagonist bicuculline via reverse microdialysis into the ventral pallidum of freely moving rats. Dialysis fractions were taken every 20 min and analyzed for dopamine and glutamate using high performance liquid chromatography. Application of propofol decreased dopamine levels in the ventral pallidum. This effect seems to be mainly mediated through GABAA receptors, since it was compensated by the GABAA receptor antagonist bicuculline. Propofol and propofol plus bicuculline exerted no

effect on glutamate release in this brain region. The reduced dopamine release in ventral pallidum was most probably mediated through a GABAergic feedback loop from the ventral pallidum via the nucleus accumbens to the dopaminergic neurons of the ventral tegmental area or by long loop feedback. As an increase but not a decrease of dopamine release in the ventral pallidum is involved in hedonic and rewarding properties, similar symptoms induced by propofol seem to be unrelated to an action of propofol in the ventral pallidum.

In vivo and *in vitro* study on the mechanism of pathogenesis of neurolathyrism

Kuniko Kusama-Eguchi[1], **Makoto Yamamoto**[1], **Tadashi Ueda**[2], **Atsuhiro Suda**[1], **Tadashi Kusama**[1], **Yoshihisa Ito**[1], **Fumio Ikegami**[3], **Kazuei Igarashi**[3], **Fernand Lambein**[4], and **Kazuko Watanabe**[1]

[1] Nihon University College of Pharmacy, Funabashi, Japan
[2] Department of Anatomy, Kanazawa Medical University, Kanazawa, Japan
[3] Graduate School of Pharmaceutical Sciences, Chiba University, Chiba, Japan
[4] Department of Biochemistry, Ghent University, Ghent, Belgium

Neurolathyrism is an upper motor neuron disease with non-progressive paraparesis of legs caused by prolonged overconsumption of grass pea seeds (*Lathyrus sativus* L.). This disease is still prevalent in several parts of the Indian subcontinent and in Ethiopia. The causative amino acid, 3-*N*-oxalyl-2,3-diaminopropanoic acid (L-beta-ODAP), a selective agonist of the 2-amino-3-hydroxy-5-methylisoxazole-4-propionate(AMPA)-type glutamate receptor, is present in this seed at a high concentration. However, the pathophysiological mechanism of neurolathyrism is not yet well understood. In this report, we describe experiments with this amino acid *in vitro* as well as *in vivo* in the rat. The acute toxicity of L-beta-ODAP on rat primary neurons was antagonized completely by the AMPA receptor antagonists but not with the *N*-methyl-D-aspartate (NMDA) antagonists. We found that L-beta-ODAP induced a rise in intracellular $[Ca^{2+}]_i$ as detected by laser confocal microscope. After a single high dose of L-beta-ODAP was administered to newborn rats, a very small amount of this compound was localized in the spinal cord and in the medulla oblongata/pons. When rats were treated with L-beta-ODAP repeatedly, symptoms similar to neurolathyrism could be observed in a few cases. In the spinal cord of these rats, the size and the number of motoneurons had changed significantly depending on the time after the treatment. Histological studies including TEM showed that L-beta-ODAP treated rats had characteristic feature of neurodegenerative diseases. These results show that L-beta-ODAP has a toxic effect on rat spinal cord probably via Ca^{2+}-permeable AMPA receptor and causes cell death after a period of time by a mechanism that needs further clarification.

Distribution of VEGFR-2 in the surrounding tissue to a minimal brain injury

J. V. Lafuente, **S. Bulnes**, and **A. Carrasco**

Department of Neurosciences, University of Basque Country, Leioa, Spain

Vascular endothelial growth factor (VEGF) is a major mediator in angiogenesis and vascular permeability. Some evidences demonstrate the involvement of its receptor flk-1 (VEGFR2) in angiogenesis, but few studies elucidate its role as mediator of the blood brain barrier (BBB) permeability. Targeting this cellular pathways present a promising alternative for the treatment of neoplasms and brain edema.

A immunohistochemical study using a panel of antibodies against flk-1 receptor was performed in adult rats following a minimal brain injury under conventional experimental surgical conditions.

Immunohistochemistry showes an increase of stained vessels, somata and processes arround the micronecrosis since 6 hours up to 72. Flk-1 was overexpressed mainly in endothelial cell, but also in astrocytes, neuronal somata and processes adjacent to the damage.

After brain injury the expression of VEGFR-2 is increased and its distribution corresponding to those of the VEGF. The whole system seem to play a significant role in disruption of the blood brain barrier (BBB). VEGF participates in the response of the CNS to injury in a dose dependent way and flk-1 is expressed in a paracrine way, so that flk-1 result in up-regulated response to brain injury.

Genomics of ionotropic glutamate receptors

Robert H. Lipsky

Section on Molecular Genetics, Laboratory of Neurogenetics, NIAAA, NIH, Rockville, Maryland, USA

Sequencing of the human, mouse, and rat genomes has enabled a comprehensive informatics approach to gene families. This approach is informative for cross-species sequence conservation related to functional conservation, within-species diversity related to functional variation and historical effects of selection, and the identification of new members of gene families. This genome informatics approach also focuses our attention on genes whose genomic locations coincide with linkages to phenotypes.

Ionotropic glutamate receptors play the largest role in excitatory neurotransmission in the brain and play central roles in a variety of behaviors such as learning that are fundamental to survival. Therefore, it is not surprising that this is a phylogenetically ancient family of receptors, being found in both animals and plants, and that this is a large gene family – there are at least 16 ionotropic glutamate receptors in the human.

We are identifying sequence variation by molecular screening technologies including dHPLC and direct sequencing, and by information mining of public (e.g. DBSNP, ENSEMBL) and private (i.e. Celera Discovery System) sequence databases. Each of the 16 known ionotropic glutamate receptors is represented in these databases, their positions on a canonical physical map (for example, the Celera map) is established, and comparison to mouse and rat sequences has been performed, revealing substantial conservation of these genes which are located on different chromosomes but found in common syntenic groups of genes. Missense variants are present in several of these receptors including NR2B, GRIA2 and GRIA3.

Human neurobehavioral phenotypes that are likely to be linked to glutamate receptor genetic variation include addictions, anxiety/dysphoria disorders, post-brain injury behavioral disorders, schizophrenia, epilepsy, learning and cognition. We are performing high throughput genotyping of candidate functional alleles and high frequency sequence variants of ionotropic glutamate receptors in order to test for association of these genes with susceptibility to different measures of neurobehavioral phenotypes.

Multiple functions of the hemeoxygenase system in the brain

Mahin Maines

Department of Biochemistry and Biophysics, Box 712, University of Rochester School of Medicine, 601 Elmwood Ave, Rochester, NY 14642, USA

The heme oxygenase (HO) system is the most effective mechanism for degradation of heme and generation of biliverdin, carbon monoxide (CO) and iron in the cell. To date, three isozymes, HO-1, HO-2, and HO-3 have been described. HO-1 and HO-2 are the catalytically active forms and have been well characterized.

Traditionally, the HO system was considered only in the context of heme degradative activity. This view has been revised within the past few years by the finding that the heme metabolites, biliverdin and CO, are biologically active molecules. While it has been suggested that overproduction of CO and free iron due to uncontrolled upregulation of the HO system may cause cytotoxicity, there is growing evidence of a role for the HO system in neuronal functions and cellular defense mechanisms. The bile pigment, biliverdin, and its reduction product, bilirubin, have been shown to be potent antioxidants. This is of particular interest since reactive oxygen species are involved in the physiological response to xenobiotics and stress and are a major contributor to an increasing number of disease states, including cancer, cerebral ischemia, amyotropic lateral sclerosis, Parkinson's and Alzheimer's disease. CO is a gaseous signaling molecule that, like nitric oxide, is believed to be involved in neuronal functions and communication.

In the central nervous system the bulk of HO activity is attributed to the glucocorticoid-responsive HO-2 isozyme, which is constitutively expressed in various neuronal populations including the olfactory bulb, pyramidal cells of the cortex and hippocampus, hippocampal granule cells, cerebellar Purkinje cells and several neurons in the brainstem and hypothalamus. Under normal conditions, HO-1 is expressed in only selected neurons in the brain including those of the dentate gyrus, ventromedial hypothalamus and brain stem. HO-1 is a stress protein and is induced in the brain by a multitude of stimuli associated with a change in intracellular redox status, including hyperthermia, cerebral ischemia, subarachnoid hemorrhage, and trauma. It has been hypothesized that upregulation of HO-1 significantly attenuates the severity of stress-related insults on physiological homeostasis and is a protective defense response to tissue injury. Therefore, the controlled upregulation of this enzyme via a pharmacological active agent might offer a novel approach to promote the cellular defense systems against neuronal deficits and stress-induced injury to the brain.

DNA breaks and up-regulation of G_1 checkpoint gene $p21^{waf1/cip1}$ in circulating leukocytes of glaucoma patients

Heike Moenkemann[1], **Josef Flammer**[2], **Kristina Yeghiazaryan**[1], **Kerstin Wunderlich**[2], **Winrich Breipohl**[3], **Hans H. Schild**[1], and **Olga Golubnitschaja**[1]

[1] Department of Radiology, and
[3] Department of Ophthalmology, Friedrich-Wilhelms-University of Bonn, Germany
[2] Ophthalmological Hospital, University of Basel, Switzerland

Objective: Vascular disorder leading to local ischemic events has been shown to play an important role in the pathophysiology of glaucomatous damage. Cell loss is a typical attribute of glaucomatous neuropathy. Although a decreased expression level of the DNA-repair XPGC-gene has been found in circulating leukocytes of normal-tension glaucoma patients, no leukopenia has been observed. Molecular mechanisms ensuring cell survival have not been elucidated yet for glaucoma with vascular disorder. Using the *ex vivo* optical imaging method of "Comet Assay" single-DNA breaks were determined in circulating leukocytes of both normal-tension and high-tension glaucoma vs. healthy controls (groups 2, 3 and 1 respectively). A relative expression of the anti-apoptotic factor $p21^{waf1/cip1}$ was investigated.

Results and conclusions: Quantification of $P21^{waf1/cip1}$ showed the highest expression rate in group 3 which was approximately 23 times higher than in the control group. which showed the lowest expression rate. The expression in group 2 was approximatelly 16 times higher than in the control group and 1,5 times lower than in group 3. These expression levels correlated well with DNA breaks measured. Since the expression of $P21^{waf1/cip1}$ in T-cells was shown to be crucial for their survival under stress conditions, we suppose further that in glaucoma accompanied with vascular disorder the up-regulation of this gene is the key event in the survival mechanisms of mononuclear blood cells. The $p21^{waf1/cip1}$ gene should be further taken into consideration as a potential marker, the up-regulation of which in T-cells of vasospastic individuals indicates an increased risk of a developing glaucoma.

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Metabotropic glutamate receptors as targets for neuroprotective drugs inexperimental parkinsonism

Giuseppe Battaglia[1], **Francesco Fornai**[1,2], **Carla L. Busceti**[1], **Francesca Biagioni**[1], **Stefano Pagano**[3], **Eugenio Parati**[3], **Valeria Bruno**[1,4], and **Ferdinando Nicoletti**[1,4]

[1] I.N.M. Neuromed, Pozzilli, Italy
[2] Department of Human Morphology and Applied Biology, University of Pisa, Italy
[3] I.N.N. "C. Besta", Milano, Italy
[4] Department of Human Physiology and Pharmacology, University of Roma "La Sapienza", Roma, Italy

Metabotropic glutamate (mGlu) receptors have emerged as promising targets in experimental parkinsonism. To examine how native mGlu2 and -3 receptors affect nigro-striatal degeneration, we used the agonist, LY379268, and the antagonist, LY341495, in mice challenged with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Systemic injection of LY379268 (1 mg/kg, i.p.) partially reduced the extent of nigro-striatal degeneration induced by MPTP ($20\,mg/kg \times 4$, i.p.), as assessed by measurements of striatal dopamine levels, striatal tyrosine hydroxylase and high affinity dopamine transporter immunostaining, and this effect was reversed by LY341495. To study the effect of mGlu receptor ligands on grafted neural stem cell (NSC) survival, dopaminergic differentiation and proper synaptic targeting, we engrafted NSCs ($500{,}000/5\,\mu l$) in the striatum of MPTP (30 mg/kg, i.p.)-treated mice. Afterwards mice were treated with LY379268, LY341495 or MPEP, a selective mGlu5 receptor antagonist. After one month, dopamine transporter-positive NSCs were detected in the striatum of engrafted animals. LY379268 did not induce any apparent effect on grafted NSCs, whereas LY341495 and MPEP inhibited their survival. To test the hypothesis that grafted NSCs were able to recover dopaminergic neurotransmission in the striatum, we used the 2-Deoxy-D-[1-^{14}C]Glucose method. Animals treated with MPTP showed an increased activity in the external glubus pallidus which was reduced by grafted NSCs. The same effect was observed in animals treated with LY379268. Treatment with MPEP inhibited the functional recovery induced by grafted NSCs. We conclude that mGlu2/3 receptors play a protective role against MPTP toxicity and that mGlu5 receptors are necessary for the survival of grafted NSCs.

New pathways to neurodegeneration in neuronal cultures

M. T. Fernández-Sánchez[1], **V. Nuñez-Nuñez**[2], **M. Pérez-Basterrechea**[1], **V. Díaz-Huerta**[3], **M. L. Fernández-Sánchez**[3], **A. Sanz-Medel**[3], and **A. Novelli**[1,2]

[1] Department of Biochemistry and Molecular Biology, University of Oviedo, E-33011 Oviedo, Spain
[2] Department of Psychology, Psychobiology, University of Oviedo, E-33011 Oviedo, Spain
[3] Department of Physical and Analitical Chemistry, University of Oviedo, E-33011 Oviedo, Spain

Excitatory amino acid receptor stimulation is coupled to a complex intracellular signaling that includes the generation of free oxygen

radicals and may possibly lead to neurodegeneration. Oxidative stress has been proposed to be the common mediator of excitatory amino acid neurotoxicity in different types of diseases, therefore the mechanisms of free oxygen radical synthesis and elimination are of particular interest. We have developed new culturing conditions that significantly increase spontaneous oxygen free radical formation in neurons in culture and induce their degeneration after 8 days in culture. Antioxidants such as vitamin E, significantly increase neuronal survival, but superoxide dismutase addition to the culture medium did not. Glutamate and sodium nitroprusside stimulation of cGMP was similar to that of cultures grown in the standard conditions, indicating a proper coupling of excitatory amino acid receptors to NOS and a proper activation of guanylate cyclase by NO. On the other hand, both nitroarginine and excitatory amino acid receptor antagonists failed in protecting neurons from spontaneous degeneration. Excitotoxicity by glutamate acting at the NMDA receptor was increased in the new culturing conditions as compared to the standard ones. Our results suggest that culturing conditions may enhance free oxygen radical production in glutamatergic neurons, independently of glutamate neurotransmission.

Alteration of amino acid neurotransmitters in hyperthermic brain injury

Hari Shanker Sharma

Department of Medical Cell Biology, Biomedical centre, Box 571, Uppsala University, SE-75123 Uppsala, Sweden

The central nervous system (CNS) is very rich in excitatory and inhibitory amino acid neurotransmitters. However their involvement in the pathophysiology of brain injury following hyperthermia is largely unknown. This investigation was undertaken to examine the possible role of excitatory amino acids, glutamate and aspartate and inhibitory amino acids, _-amino butyric acid (GABA) and glycine in hyperthermia induced brain pathology. Subjection of conscious young rats to heat stress (HS) at 38°C in a biological oxygen demand (BOD) incubator induced marked redistribution of these amino acids. Thus, at 1 h after HS glutamate, aspartate, GABA and glycine increased (6–10 fold) in cerebral cortex, cerebellum and brain stem and a 2–4 fold decrease in glutamate and GAB in the hippocampus and spinal cord. The aspartate and glycine levels remained unchanged. At 2 h after HS, all these amino acids were elevated in most of the brain regions. Interestingly, a marked decrease in GABA and glycine levels (4–6 fold) were observed at the end of 4 h after HS. The glutamate and aspartate levels were decreased in the cerebellum and the spinal cord, whereas, hippocampus and brain stem showed a mild increase. Pretreatment with neuroprotective drugs p-CPA (a serotonin synthesis inhibitor), indomethacin (a prostaglandin synthase inhibitor) or naloxone (an opioid receptor antagonist) resulted in a profound elevation of inhibitory amino acids (4–8 fold) in many brain regions at 4 h after HS. These drugs are able to inhibit the elevation of the excitatory amino acids at this time. These observations suggest that a balance between excitatory and inhibitory amino acids in the CNS is crucial for brain damage in hyperthermia.

Neuroprotective effects of melanocortins in spinal cord injury

A. Skottner[1], **H. S. Sharma**[2], **T. Lundstedt**[1], **M. Flärdh**[1], **J. Westman**[2], and **C. Post**[1]

[1] Melacure Therapeutics AB, Uppsala, Sweden
[2] Department of Medical Cell Biology, Laboratory of Neuroanatomy, Box 571, Biomedical Centre, Uppsala University, Uppsala, Sweden

Melanocortin peptides such as _-melanocyte stimulating hormone (_-MSH) and adrenocorticotropin (ACTH) or its fragments exert beneficial effects on functional recovery from injured peripheral and central nervous tissues. Local administration of melanocortin peptides improves functional recovery of peripheral and brain tissues. This indicates that melanocortin peptides are effective at the lesion site.

In the rat brains, MSH receptor (MC1-R), ACTH receptor (MC2-R), MC3-R and MC4-R has recently been isolated and characterized. These multiple melanocortin receptors play some role in neurotrophic or neuroprotective effects in the brain following injury. Melacure has developed several low molecular weight, non-peptide compounds with varying affinity and selectivity on the melanocortin receptors. The efficacy of some of these compounds was evaluated on a focal spinal cord trauma induced edema formation, breakdown of the blood-spinal cord barrier and cell injuries in a rat model.

Five new Melacure low molecular weight compounds ME10092, ME10354, ME10393, ME10431 and ME10501 were examined for their possible neuroprotective effects in the spinal cord injury (SCI). The compounds were chosen based on their different affinities to the melanocortin receptors. Each compound was dissolved in saline and tested at 3 different doses, i.e. 1 μg, 5 μg and 10 μg total dose in 10 μl applied topically 5 min after SCI. The animals allowed to survive 5 h.

A focal trauma to the rat spinal cord made by an incision on the right dorsal horn (T10-11) resulted in profound edema formation, leakage of Evans blue albumin and cell injury in the T9 segment at 5 h. Topical application of ME10501 in high doses (10 μg) resulted in most significant neuroprotection in the T9 and T12 segments of the cord compared to other compounds. On the other hand, only a mild or no effect on visual swelling, spinal cord water content and Evans blue extravasation was observed in ME10092, ME10354, ME10393 and ME10431 treated and traumatised rats given at 3 different doses. These observations suggest that non-peptide compounds with affinity to Melanocortin receptors are capable to induce neuroprotective in spinal cord following trauma.

Age-related changes in kainate receptor-mediated excitotoxicity

R. A. R. Tasker[1], **T. A. Doucette**[2], **D. S. Kerr**[4], **M. Sweeney-Nixon**[3], and **C. L. Ryan**[2]

[1] Departments of Biomedical Sciences, and
[2] Department of Psychology and
[3] Department of Biology, University of Prince Edward Island, Charlottetown, PEI, Canada
[4] Department of Pharmacology, University of Otago, Dunedin, New Zealand

Kainate receptors are primarily responsible for excitotoxic neuronal cell death following exposure to either kainic acid or domoic acid in a variety of animal models. Several studies have also implicated NMDA receptors and metabotropic glutamate receptors in domoic acid induced neurotoxicity. It is also well established that both neonatal and aged rats, as well as aged humans, are more susceptible to the neurotoxic actions of both compounds *in vivo*. What is not established, however, is whether increased sensitivity to the neurotoxic properties of these compounds is due to age-related alterations in the sensitivity of kainate receptors to ligand-mediated toxicity, or results from changes in the pharmacokinetic properties of these drugs in young and aged animals or patients. The current study was designed to evaluate the neurotoxic properties of both domoic acid and kainic acid using *in vitro* preparations derived from both neonatal, normal adult, and aged adult rats. The excitoxic properties of both drugs were evaluated alone and in the presence of various receptor-selective antagonists to determine the relative contribution of different

kainate receptors to domoic and kainic acid mediated toxicity. Results indicate that there are age-related changes in kainate receptor-mediated toxicity that are potentially relevant to understanding the role of kainate receptors in both brain development and the susceptibility of the aged brain to naturally-derived toxins.

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Expression of ABC-1 transporter is elevated in human glioma cells under temozolomide treatment and irradiation

Daniela B. Trog, Heike Mönkemann, Nikolai Härtel, Heinrich Schüller, Hans H. Schild, and **Olga Golubnitschaja**

Department of Radiology, Friedrich-Wilhelms-University of Bonn, Germany

Objective: Application of chemotherapeutic treatment on glioma patients has minor success. Little is known about mechanisms of a cross-resistance of gliomas towards actual therapies, yet. ABC-1 belongs to the group of transporters known to be involved in the export of anticancer drugs conveying resistance of cancer cells towards chemotherapy. This study investigates an effect of both temozolomide (TMZ)-treatment and/or irradiation on the expression of the ABC-1 transporter in human U87-MG glioma cells.

Material and methods: In parallel experiments U87-MG cells underwent either irradiation, chemo-treatment using TMZ, and combined chemo/radiation treatment. After each treatment the cells were incubated either 2 or 24 hours at 37°C and counted before protein analysis using Western-Blot technique.

Results and conclusions: ABC-1 was constituently expressed in both treated and untreated U87-MG cells. The expression increased spontaneously correlating with the cell density. Within 2 hours after TMZ-treatment it was considerably up-regulated in a dose dependent manner. Irradiation had a comparable or even higher inducible effect on the ABC-1 expression rate depending on cell density. The highest expression rates were observed in cultures with high cellular density 2 hours after application of the combined treatment. This effect decreased partially within 24 hours after treatment, but was still well detectable. Strong up-regulation of ABC-1 expression under both irradiation and chemo treatment might be a clue to the multidrug and irradiation cross-resistance mechanisms of malignant glioma cells converting the ABC-1 transporter to an attractive pharmacological target for a clinical breakthrough in the therapy of malignant gliomas.

Neuroprotective effects of antioxidants on heat shock protein (HSP 72 kD) and the blood-brain barrier in hyperthermic brain injury

Jan Westman

Laboratory of Neuroanatomy, Department of Medical Cell Biology, Biomedical Centre, Box 571, Uppsala University, SE-751 23 Uppsala, Sweden

Hyperthermia exceeding 41°C induces profound cellular stress and generation of free radicals. It seems likely that free radicals contribute to the BBB dysfunction by directly or indirectly damaging the cerebral endothelium. In present experiments, influence of several potent antioxidants, H-290/51, extract of Gingko biloba (EGB-761) and one of its active component, Gingkolide B (BN-520 21) was examined in hyperthermia induced BBB breakdown and heat shock protein (HSP 72 kD) expression in a rat model. Subjection of conscious young rats (100–150 g) to heat exposure (38°C) in a biological oxygen demand (BOD) incubator for 4 h (relative humidity 45–47%, wind velocity 20–25 cm/sec) resulted in marked extravasation of Evans blue and radioactive iodine ($^{[131]}$I-sodium) in several brain regions. These brain areas are also associated with profound upregulation of HSP expression. Pretreatment with H-290/51 (50 mg/kg, p.o. 30 min before HS; EGB-761 (50 mg/kg, p.o. for 5 days) or BN-52021 (2.5 mg/kg, p.o. for 5 days) significantly reduced the extravasation of Evans blue and iodine tracers in the brain. In these drug-treated rats, expression of HSP was much less evident compared to the untreated heat exposed animals. These effects were most pronounced in the rats treated with H-290/51 pretreatment compared to EGB-761. Taken together, these observations suggest that free radicals play an important role in hyperthermia induced BBB dysfunction and HSP induction.

L-cysteine desulfuration in various human and mouse brain regions

Maria Wróbel, Jerzy Czubak, and **Halina Jurkowska**

Institute of Medical Biochemistry, Jagiellonian University Medical College, 31-034 Kraków, ul. Kopernika 7, Poland

L-cysteine desulfuration provides reactive sulfane sulfur atoms for detoxification reactions and the synthesis of iron-sulfur proteins, affecting protein function and participation in antioxidative processes. The activity of enzymes participating in the formation and metabolism of sulfane sulfur compounds, i.e.: rhodanese, 3-mercaptopyruvate sulfurtransferase (MPST), cystathionine γ-lyase (CST), and the level of sulfane sulfur compounds were investigated in various regions of human and mouse brain.

Only a trace activity of CST was found in all the investigated regions of the human brain in specimens collected postmortem. The MPST activity, in turn, was relatively high in the human brain – up to $23 \pm 1\,\mu$moles of product $\cdot \min^{-1} g^{-1}$ in the cerebellum. The value of rhodanese activity varied greatly (up to $4.1 \pm 0.1\,\mu$moles of product $\cdot \min^{-1} g^{-1}$ in the cerebellum). It seems that in the human brain cysteine desulfuration via the MPST reaction is the main pathway of sulfane sulfur generation, the highest level of which was determined in the hippocampus.

Histochemical examination of rhodanese activity in cryostat sections from various mouse brain regions showed that the sites of rhodanese activity were ubiquitous throughout the brain. They were revealed as punctuate, granular, dark dots (hippocampus), or as long, threadlike particles (especially abundant in the region of the telencephalon in the astroglia cells). There were sites with a high density of the histochemical test products, for example the ependymoma of the fourth cerebral ventricle, choroid plexus and nerve ducts.

Based on the above results we may conclude that the intensity of L-cysteine desulfuration depends on the brain region.

Increased expression of ABC-transporters in mononuclear blood cells of glaucoma patients and vasospastic individuals indicates the common feature of up-regulated multidrug resistance

Kristina Yeghiazaryan[1], **Josef Flammer**[2], **Hans H. Schild**[1], and **Olga Golubnitschaja**[1]

[1] Department of Radiology, Friedrich-Wilhelms-University of Bonn, Germany

[2] Ophthalmological Hospital, University of Basel, Switzerland

Objective: Glaucoma is a systemic neurodegenerative disease. Since vascular disregulation is supposed to be a risk factor for the development of glaucomatous damage, a preventive medicamentous treatment might slow down the disease development. Application of therapeutic treatment on glaucoma patients is not effective enough yet. This efficiency depends on a specificity of drug delivery and drug resistance of target cells. Non-invasively, in mononuclear blood cells we investigated *ex vivo* a potential multidrug resistance of both normal-tension and high-tension glaucoma

patients, and that of non-glaucomatous vasospastic individuals vs. healthy individuals (groups 3, 4, 2 and 1 respectively). As a ''screening'' method of Transcriptomics ''Expression Array'' was applied for the analysis of differential gene expression. Protein samples underwent a Western-blot quantitative analysis using specific anti-bodies against selected human ABC-transporters in order to confirm the up-regulation of these genes also on the protein level.

Results and conclusions: ABC-1 and MDR-3 transporters were shown to be highly up-regulated on both transcriptional and translational levels in groups 2, 3 and 4 compared to the control group. Both transporters act as energy-dependent unidirectional transmembrane drug efflux pump to reduce intracellular drug accumulation. Both of them can transport a wide range of hydrophobic drugs, and may see lipid analogous as just another drug. Since no indications were found for a tissue-specific regulation of alternative splicing of pre-mRNA of both transporters, this finding indicates that multidrug resistance might be generally increased in both glaucoma patients and vasospastic individuals being the common part of pathophysiological mechanisms.

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NO/Arginine

L-Arginine metabolism directed to L-ornithine via arginase I induction favors the survival and replication of *leishmania* inside macrophages

I. Corraliza

Department of Biochemistry & Mol. Biol., Fac. of Veterinary, Univ. of Caceres, Spain

Leishmania spp. are *protozoan* parasites that invade and replicate inside macrophages. In the established *Leishmania major* model of infection, activated macrophages metabolize arginine preferentially by two alternative pathways; either is hydrolyzed to ornithine and urea by the inducible arginase I, or is metabolized by iNOS to hydroxy-L-arginine, citrulline and nitric oxide (NO).

The type of T cell response triggered by the parasite, competitively regulates the balance between these two enzymes: If the animal is able to generate a strong Th1 response, iNOS is induced and the replication of parasites can be controlled. However, if an unbalanced Th2 response is the predominant, the animal succumbs to the infection.

We have investigated the dependence of *Leishmania* infection on arginase I induction, triggered by the th2 cytokines IL-4 and Il-10 as well as TGF-betta.

Treatment of *L.major*-infected BALB/c macrophages with all of these cytokines led to a proportional increase in the number of intracellular amastigotes. Moreover, parasite proliferation and arginase activity levels were significantly higher in cells from the susceptible BALB/c mice than in those from the resistant C57BL/6 mice, indicating that a strong correlation exist between the permissibility of host cells to the parasite and the induction of arginase I in macrophages.

Specific inhibition of arginase by N-hydroxy-nor-L-arginina (nor-LHOA) reverted the growth, while L-ornithine, the product of arginase reaction and putrescine promoted parasite proliferation.

These results strongly suggest that the parasite cell division depend critically on the level of L-ornithine available in the host, which is used by *leishmania* to generate polyamines needed to proliferate.

The interplay of arginases and iNOS activities in human skin: lessons learned from psoriasis

Victoria Kolb-Bachofen, Oliver Schnorr, Daniela Bruch-Gerharz, Thomas Ruricka, and **Christoph Suschek**

Research Group Immunobiology and Department of Dermatology, Med. Fac., Heinrich-Heine-University, Duesseldorf, Germany

The proinflammatory skin disease psoriasis is characterized by abnormally high proliferation of keratinocytes and by a ''constitutive'' expression of inducible NO synthase (iNOS) in all lesions. Contradictory to this phenotype, we had shown earlier that nitric oxide (NO) at high concentrations inhibits keratinocyte proliferation and induces differentiation *in vitro*. Although high-output NO synthesis is suggested by the expression of iNOS, the pronounced hyperproliferation of psoriatic keratinocytes indicates that iNOS activity is too low to effectively deliver antiproliferative signals.

Here we show that arginase 1 (ARG1), which participates in the regulation of iNOS activity by competing for the common substrate L-arginine, is highly overexpressed in the hyperproliferative psoriatic epidermis and co-expressed with iNOS. Treatment of primary cultured keratinocytes with Th1-cytokines, as present in a psoriatic environment, leads to *de novo* ex-pression of iNOS but concomitantly a significant downregulation of ARG1. Persistent ARG1 overexpression in psoriasis lesions, therefore, may represent an disease-associa-ted deviation from normal expression patterns. Furthermore, the culturing of activated keratinocytes in the presence of an ARG inhibitor results in a twofold increase in nitrite accumulation providing evidence for an L-arginine substrate competition in human keratinocytes. High-output NO synthesis is indeed associated with a significant decrease in cellular proliferation as shown by downregulation of Ki67 expression in cultured keratinocytes but also in short-term organ cultures of normal human skin. In summary, our data demonstrate for the first time a link between a human inflammatory skin disease, limited iNOS activity and ARG1 overexpression.

Asymmetrical dimethylarginine (ADMA): endogenous NOS-inhibitor, cardiovascular risk factor and explanation for effects of L-arginine *in vivo*

R. Maas and **R. Böger**

Institut für Experimentelle und Klinische Pharmakologie, Universitätskrankenhaus Hamburg-Eppendorf, Hamburg, Germany

The L-arginine-nitric oxide pathway is involved in the regulation of diverse physiological functions including endothelium-dependent vasodilation, thrombocyte aggregation, neurotransmission, immune defense, apoptosis and erectile function. Accumulating evidence is linking asymmetrical dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthases (NOS), to human disease. ADMA is formed endogenously by degradation of proteins containing arginine residues that have been methylated by S-adenosylmethionine-dependent methyltransferases (PRMTs). There are two major routes of elimination: renal excretion and enzymatic degradation by dimethylarginine dimethylaminohydrolase (DDAH I and II). Elevated plasma ADMA concentrations are found in various clinical settings ranging from renal failure to atherosclerosis, hypertension and diabetes. Moreover, in patients with cardiovascular or renal disease elevated plasma ADMA concentrations independently predict progression of atherosclerosis and mortality. So far, causality in cardiovascular disease is considered likely but not definitely proven: Infusion of ADMA ameliorates endothelium-dependent vasodilation in humans, and the fact that life has evolved a highly specific enzymatic mechanism for its degradation (i.e. control) provides further evidence for a direct (patho-)physiological role of ADMA. With the exception of decreased excretion in renal failure, mechanisms leading to elevated

plasma ADMA concentrations (i.e. possible targets for therapeutic interventions) are poorly understood. Up-regulation of PRMTs (by oxLDL), gene polymorphisms of DDAH or down-regulation of DDAH activity (by homocysteine) have been suggested as possible mechanisms. First studies are under way to determine whether beneficial effects of some cardiovascular drugs or L-arginine can be attributed to modification of ADMA metabolism or ADMA effects.

Disturbed L-arginine homeostasis in the pathophysiology of allergic asthma

H. Meurs, H. Maarsingh, S. McKay, J. de Boer, M. Schuiling, and **J. Zaagsma**

Department of Molecular Pharmacology, University Centre for Pharmacy, A. Deusinglaan 1, 9713 AV Groningen, The Netherlands

Allergic asthma is an inflammatory disease, characterized by allergen-induced early (EAR) and late (LAR) asthmatic reactions, eosinophilic airway inflammation, and airway hyperresponsiveness (AHR) to bronchoconstricting stimuli, which develops both after the EAR and LAR. Using a guinea pig model of allergic asthma, we demonstrated that a deficiency of epithelial cNOS-derived, bronchodilating NO contributes to a major extent to the AHR observed after the allergen-induced EAR. This NO deficiency is caused by limitation of L-arginine, the substrate for cNOS, which may involve at least two mechanisms: (1) inhibition of epithelial uptake of L-arginine through cationic amino acid transporters induced by polycations, including eosinophil-derived major basic protein, and (2) increased activity of arginase, which may compete with cNOS for their common substrate. iNOS is induced in the airways during the LAR. The development of AHR after the LAR appears to involve the formation of the pro-inflammatory and pro-contractile oxidant peroxynitrite from iNOS-derived NO and superoxide anions. Remarkably, the AHR after the LAR could be reduced by the polycation antagonist heparin, by the arginase inhibitor N^{ω}-hydroxy-nor-L-arginine, and by suppletion of L-arginine, indicating that polycation- and arginase-induced deficiency of L-arginine also contribute to iNOS-induced pathology. This finding may well be explained by previous observations that at low L-arginine concentrations, iNOS produces both NO and superoxide anions, resulting in a very efficient formation of peroxynitrite. In conclusion, a (relative) shortness of both cNOS- and iNOS-derived NO, due to altered L-arginine homeostasis, is a major causative factor in the pathology of asthma.

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A novel pathway of NO-mediated apoptosis

Masataka Mori, Seiichi Oyadomari, and **Tomomi Gotoh**

Department of Molecular Genetics, Kumamoto University School of Medicine, Kumamoto 860-0811, Japan

Nitric oxide (NO) is a messenger molecule functioning in vascular regulation, immunity, neurotransmission and others, and has been implicated in many diseases. NO is synthesized from arginine by NO synthase (NOS), and the availability of arginine has been shown to be a rate-limiting factor in NO production. Citrulline formed as a by-product of the NOS reaction can be recycled to arginine by argininosuccinate synthetase and argininosuccinate lyase, forming the ''citrulline-NO cycle''. On the other hand, arginase isoforms have been shown to down-regulate NO production by depleting arginine. Excessive NO leads to apoptosis in various cells. When macrophages were immunostimulated or treated with NO, apoptosis occurred. Under these conditions, p53 accumulation was not observed, indicating that DNA damage is not the main trigger of NO-mediated apoptosis. Furthermore, apoptosis was induced in p53-deficient microglial cells by NO. We found that CHOP/GADD153, a C/EBP family transcription factor which is involved in ER stress-mediated apoptosis, is induced. The induction of CHOP was followed by the mitochondrial apoptotic pathway involving cytochrome *c* release and activation of caspase cascade. Excessive NO production in cytokine-activated β-cells has been implicated in β-cell distruption in type 1 diabetes. NO depleted ER Ca^{2+}, and overexpression of calreticulin, a major Ca^{2+} binding protein in ER, increased ER Ca^{2+} and protected cells against NO-mediated apoptosis. Furthermore, pancreatic islets from CHOP-deficient mice showed resistance to NO. We conclude that NO depletes ER Ca^{2+}, causes ER stress, and leads to apoptosis.

Protective action of exogenous L-arginine against stress-induced gastric mucosal lesions in rats

Yoshiji Ohta[1] and **Keiji Nishida**[2]

[1] Department of Chemistry, School of Medicine, Fujita Health University, Toyoake, Japan

[2] Department of Internal Medicine, Daido Hospital, Nagoya, Japan

Nitric oxide (NO) produced from L-arginine by NO synthase is known to inhibit neutrophil-endothelial cell adhesion and to stimulate gastric mucus synthesis and secretion. We have reported that neutrophil infiltration and a decrease in gastric mucus level are involved in gastric mucosal lesion development in rats with water immersion restraint stress and that pre-administered L-arginine protects against the stress-induced gastric mucosal lesions through NO produced via constitutive NO synthase. However, the exact protective action of exogenous L-arginine against gastric mucosal lesions in rats with water immersion restrain stress is still unclear. Therefore, we examined the protective action of exogenous L-arginine against gastric mucosal lesions in rats with water immersion restraint stress. Wistar rats were subjected to water immersion restraint stress for 3 or 6 h. L-Arginine, D-arginine or L-arginine with either L-NMMA, an inhibitor of NO synthase, or D-NMMA was injected to rats 0.5 h before stress induction. Pre-administered L-arginine, but not D-arginine, protected against gastric mucosal lesions with attenuation of increased myeloperoxidase activity and decreased hexosamine and adherent mucus levels in the gastric mucosa 3 or 6 h after stress induction. The L-arginine-mediated protection against gastric mucosal lesions and attenuation of increased myeloperoxidase activity and decreased hexosamine and adherent mucus levels in the gastric mucosa were counteracted by co-administration of L-NMMA, but not D-NMMA. These results suggest that exogenous L-arginine protects against gastric mucosal lesions in rats with water immersion restraint stress by inhibiting neutrophil infiltration into the gastric mucosa and by preserving gastric mucus synthesis and secretion.

Enzymes of arginine/NO and ADMA pathways in endothelial cells treated with homocysteine

G. G. Pinna[1], **A. Naitana**[1], **A. Pantaleo**[1], **A. Manunta**[2], and **M. Ganadu**[2]

[1] Department of ''Scienze Biomediche, sezione di Biochimica'', University of Sassari and

[2] ''Servizio Nefrologia e Dialisi'', ASL 1 Sassari, P.O. Ozieri, Italy

Endothelial cells synthesize the vasodilator substance nitric oxide (NO), which causes relaxation of vessel's smooth muscular cells, from L-arginine by acatalysis of NO synthase (ecNOS). The impairment in the production and/or the bioavailability of nitric oxide is defined as endothelial dysfunction, a situation found in cardiovascular diseases, diabetes, hyperhomocysteinemia, preeclampsia and end-stage renal disease. The aminoacid L-arginine can be metabolized also by the enzyme arginase, with the production of urea and ornithine, that can lead to polyamine and/or proline synthesis. Thus, an increased utilization of L-arginine by arginase would parallel a decreased ecNOS activity and

NO production. The ecNOS is also under the control of an endogenous inhibitor, asymmetric dimethylarginine (ADMA), which forms following post-translational dimethylation of arginine residues in proteins by action of a methyltransferase (PRMT) and subsequent proteolysis. ADMA can in turn be inactivated by a dimethylarginine dimethylaminohydrolase (DDAH).

We have induced endothelial dysfunction in a line of immortalized HUVEC (Human Umbilical Vein Endothelial Cells) by treatment with L-homocysteine (HCys) 0,5–3 mM for 24 hrs. Progression of endothelial dysfunction was monitored by LDH activity increase in culture medium. Homogenates of harvested cell were assayed for the following enzymatic activities: ecNOS, arginase, PRMT and DDAH. Our preliminary results show that ecNOS activity and NO production are sligthly decreased, DDAH is decreased by nearly 50%, while arginase and PRMT activities are both increased by more than 60%. Time course experiments as well as ADMA determination in cell extracts and medium will help in understanding temporal effects of homocysteine action on enzyme activities involvrd in NO and ADMA metabolisms.

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IL-4 and IL-13 up-regulate arginase in rat primary airway fibroblasts

K. Racké, D. Lindemann, and **M. Fuhrmann**

Institute of Pharmacology & Toxicology, University of Bonn, Reuterstraße 2b, D-53113 Bonn, Germany

We have previously shown that arginase can limit L-arginine availability for NO synthesis. Moreover there is evidence that an increased arginase activity plays a role in the development of allergen-induced airway hyperresponsiveness. Since fibroblasts could be a cellular compartment in which arginase is up-regulated during allergic reactions, it was tested whether Th2 cytokines affect arginase in rat airway fibroblasts.

Primary airway fibroblasts (passage 3–4) obtained from isolated rat trachea by an outgrowth technique were used. Cells ($0.7^{*}10^{6}$ cells/well) were cultured for 2 days in presence of 5% or 10% FCS followed by up to 40 h in additional presence or absence of IL-4 (10 ng/ml) or IL-13 (10 ng/ml). Arginase activity in fibroblasts cultured under control conditions in presence of 5% or 10% FCS amounted to 6.0 ± 1.7 and 20.4 ± 1.6 mU/μg protein, respectively (mean $\pm$ SEM, $n > 20$). Using RT-PCR, mRNA for arginase I was clearly detected, whereas mRNA for arginase II was hardly detected. Presence of IL-4 or IL-13 (40 h) caused an increase in arginase activity to 25.9 ± 1.9 and 27.7 ± 2.8 mU/μg protein respectively (5% FCS) and to 33.3 ± 1.6 and 39.1 ± 2.9 mU/μg protein, respectively (10% FCS). IL-4 and IL-13 caused a clear increase in mRNA for arginase I and a minor, more variable increase of arginase II mRNA.

In conclusion, IL-4 and IL-13-induced up-regulation of arginase activity in airway fibroblast could play a role in the pathophysiology of allergic airway diseases.

Characterization and NO-production mechanism of *Hyphomicrobium* nitrite reductase containing two type 1 and one type 2 coppers

Shinnichiro Suzuki, Kunishige Kataoka, Kazuya Yamaguchi, Mayuko Kobayashi, and **Koushi Itoh**

Department of Chemistry, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan

There are some kinds of enzymes, which catalyze the production of NO. Dissimilatory nitrite reductase (NIR) located in the periplasm of denitrification bacteria catalyzes one-electron reduction of nitrite to NO. The Cu-containing NIRs from *Achromobacter cycloclastes* (green AcNIR) and *Alcaligenes xylosoxidans* (blue AxNIR) have the similar trimer structures, in which a monomer (ca. 37 kDa) contains a type 1 Cu and a type 2 Cu. The type 1 Cu plays the role of electron-transfer and the type 2 Cu is the catalytic reduction site of the substrate.

We first found that blue-green *Hyphomicrobium denitrificans* NIR (HdNIR, ca. 50 kDa) shows unique spectroscopic features of the type 1 Cu, compared with those of AcNIR and AxNIR: the visible absorption spectrum implies that the enzyme has two kinds of the type 1 Cu. The genetic analysis of HdNIR indicated that the polypeptide is consist of blue copper protein-like domain (ca. 14 kDa) containing one type 1 Cu ligand motif and NIR-like domain (ca. 35 kDa) containing one type 1 Cu and one type 2 Cu ligand motifs. HdNIR has been successfully proteolyzed to two protein fragments (14 kDa and 35 kDa) with subtilisin. The visible absorption, CD, EPR spectra of these proteins imply that the blue 14-kDa protein fragment has one type 1 Cu site, which is axially elongated trigonal bipyramidal, and the green 35-kDa protein fragment has one type 1 Cu site having a flattened tetrahedral geometry with one type 2 Cu site. The 35-kDa fragment shows the nitrite reduction activity a little higher than to that of the native enzyme. Moreover, the reaction mechanism of HdNIR will be discussed.

Plant Amino Acids

The molecular basis of quality protein maize

Brian A. Larkins

Department of Plant Sciences, University of Arizona, Tucson, AZ 85721, USA

Maize *opaque2* (*o2*) mutants have an increased level of free and protein-bound lysine, but they have a soft, starchy endosperm that prevented the development of high lysine corn. The identification of genes that modify the *o2* phenotype, resulting in a hard, vitreous kernel, led to the development of Quality Protein Maize (QPM), a high lysine corn with a normal phenotype. Our research has focused on the mechanism by which *o2* increases the lysine content of the endosperm and how *o2* causes a soft, starchy endosperm. One of the lysine-rich proteins increased in *o2* mutants is elongation factor 1α (eEF1A). eEF1A contains 10% lysine, but it only accounts for about 2% of the lysine in the endosperm. There is a high correlation ($r = 0.9$) between the concentration of eEF1A and protein-bound lysine in the endosperm, which could be explained by the relationship between eEF1A and the cytoskeleton; we found eEF1A to be complexed with F-actin in a network surrounding the rough endoplasmic reticulum at sites where protein bodies are forming. QTLs associated with the high free lysine content of *o2* mutants identified several loci, including one that encodes *Ask2*, a lysine-sensitive aspartate kinase. The reduction in α-zein synthesis in *o2* mutants results in small protein bodies. Changes in protein body size also occur in *floury2* (*fl2*), a mutation that prevents cleavage of the signal peptide of a 22-kD α-zein, leading to disrupted protein body assembly. In *o2* and *fl2*, as well as several other opaque mutants, there is a significant up-regulation of genes associated with stress, similar to the ''misfolded protein'' response. Our understanding of these pleiotropic effects, as well as how *o2* modifiers ameliorate this response is limited, but recent studies show that *o2* modifiers are associated with changes in protein body formation and starch structure.

Lysine improvement in the seeds of pigeonpea

Tran Thanh Thu, Eric Dewaele, Le Quang Trung, Michel Jacobs, and **Geert Angenon**

Laboratory of Plant Genetics, Vrije Universiteit Brussel (VUB), Pleinlaan 2, B-1050 Brussel, Belgium

Pigeonpea (*Cajanus cajan L.*) is a major grain legume crops of the tropics and subtropics. Although pigeonpea seed protein content is relatively high, its nutritional value is limited due to a deficiency in the essential amino acids such as cysteine, methionine and lysine.

In higher plants lysine is biosynthesised through an aspartate-derived pathway and the key enzyme of lysine biosynthesis, dihydrodipicolinate synthase, is feedback-regulated by lysine. The strategy for lysine improvement in pigeonpea is based on a mutated gene encoding dihydrodipicolinate synthase insensitive to feedback inhibition. Constructs containing the mutated gene under the control of the phaseolin or 2S2 promoter were introduced into pigeonpea via *Agrobacterium* or particle bombardment. Among fifteen analysed transgenic lines, twelve showed 2 to 5 fold enhanced enzyme activity in immature seeds compared to wild type plants. Assays of the lysine degradative enzyme lysine ketoglutarate reductase, showed no co-ordinated regulation of lysine biosynthesis and catabolism during pigeonpea seed maturation. The content of free lysine in mature seeds has been determined in 10 transgenic lines and 6 of them showed lysine enhancement from 1.6 to 8.5 times compared with wild type. The lysine enhancement seems to affect the level of other amino acids related to the aspartate pathway in the transgenic seeds. Threonine and methionine tend to decrease slightly. However the line with an 8.5 fold increase in lysine, the content of methionine and threonine and some other amino acids are remarkably increased whereas the phenylalanine content was dramatically reduced (17 times). The seeds of all transgenic lines are morphologically normal and fertile.

Characterization of storage proteins of maize endosperm mutants

A. A. Toro[1], **C. M. Bellato**[2], **L. W. Meinhardt**[3], **F. Mestrinelli**[2], **B. D. A. Berdejo**[1], **P. J. Lea**[4], and **R. A. Azevedo**[1]

[1] Departamento de Genética, ESALQ-USP, Brazil
[2] CENA-USP, Brazil
[3] UNICAMP, Brazil
[4] Lancaster University, UK

Cereal seeds are an excellent source of carbohydrates, but the storage proteins are poor in some essential amino acids, mainly lysine. Storage proteins from several wild-type, opaque mutants and floury mutants (B37+, B37*o7*, B77/79+, B77/79*o5*, Oh43+, Oh43*o1*, Oh43*o2*, Oh43*fl1*, Oh43*fl2*, W22+, W22*o10*, W22*o11* and W22*o13*) were separated into five protein fractions (albumins, globulins, glutelins, zein 1 and zein 2). These protein fractions were analysed by SDS-PAGE. Albumin, globulin, glutelin and zein storage proteins showed a high degree of variability between the inbred lines Oh43, W22, B37 and the hybrid line B77/79. Due to the variability observed, the mutants lines could not be directly compared. Therefore, all comparisons were made between the mutants and their respective progenitor lines. The levels of zein 1 were generally lower than their respective backgrounds in all of the opaque and floury mutants evaluated except for B37*o7*. For zein 2, W22*o10* and W22*o13* were the only mutants that showed higher levels when compared to their backgrounds. Albumin levels in the mutants were always lower than in their respective wild-type backgrounds. Globulin and glutelin levels varied depending on the mutant type and background, and depending on the mutation, proteins appeared or disappeared in relation to the wild-type. The results indicated a large variability among the mutants, which may help to explain the differences observed in the concentration of lysine in relation to the wild-type maize endosperms. (Financial support from FAPESP, Brazil, and the British Council)

Effect of cadmium treatment on soluble amino acids concentration and the activitiy of antioxidant enzymes in plants

P. L. Gratão[1], **G. J. G. Pereira**[2], **R. A. Gomes-Junior**[1], **F. R. Capaldi**[3], **R. F. Fornazier**[1], **R. R. Ferreira**[1], **P. F. Cardoso**[1], **S. Filipi**[2], **A. P. Vitória**[4], **R. J. Smith**[5], **P. J. Lea**[5], **V. A. Vitorello**[3], **P. Mazzafera**[2], and **R. A. Azevedo**[1]

[1] Departamento de Genética, ESALQ-USP, Brazil
[2] UNICAMP, Brazil
[3] CENA-USP, Brazil
[4] UENF, Brazil
[5] Lancaster University, U.K.

Heavy metals can be highly toxic. Cadmium (Cd) occurs in low concentrations in soils but the concentration may be high in areas that have been subjected to mining or application of sewage sludge. Cd enters the plant system rapidly where it accumulates mainly in the root system leading to plant growth inhibition. Cd can also induce the production of reactive oxygen species (ROS). We studied the antioxidant responses of plants (tobacco, coffee, rice, radish, soybean, crotalaria and sugarcane) to Cd exposure. Seedlings and *in vitro* cell cultures were grown in increasing concentrations of $CdCl_2$, ranging from 0.01–3 mM, for up to 96 h. Analysis of Cd uptake indicated that most of the Cd accumulated in the roots, but some was also translocated to the leaves. Roots and leaves were analysed for catalase (CAT), glutathione reductase (GR) and superoxide dismutase (SOD) activities. GR activity increased considerably in the roots of all plant species exposed to Cd. CAT activity increased in roots but to a much lesser extent when compared to GR, and varied depending upon the plant species. SOD activity staining revealed several isoenzymes in the leaves of all plant species, however, only in radish was there a clear increase in enzyme activity. The results suggest that the main response may be via the activation of the ascorbate-glutathione cycle for the removal of hydrogen peroxide, or to ensure the availability of reduced glutathione for the synthesis of Cd-binding proteins. (Financial support from FAPESP, Brazil, and the British Council)

Lysine metabolism in sorghum

R. R. Ferreira[1], **R. F. Fornazier**[1], **S. A. Gaziola**[2], **L. W. Meinhardt**[3], **P. J. Lea**[4], and **R. A. Azevedo**[2]

[1] UNICAMP, Brazil
[2] Departamento de Genética, ESALQ-USP, Brazil
[3] CENA-USP, Brazil
[4] Lancaster University, U.K.

Cereal seeds contain variable amounts of storage proteins, but in general these proteins are of poor quality due to the presence of low concentrations of lysine, tryptophan and threonine. It is important to increase our understanding of the regulation of lysine metabolism, in order to improve the nutritional quality of plants by genetic manipulation. Among the enzymes involved in lysine metabolism, aspartate kinase and dihydrodipicolinate synthase are important in lysine biosynthesis, whereas lysine 2-oxoglutarate reductase and saccharopine dehydrogenase play a key role in lysine catabolism. We have isolated and partially purified by ion exchange and gel filtration chromatography, aspartate kinase, homoserine dehydrogenase, lysine 2-oxoglutarate reductase and saccharopine dehydrogenase from sorghum seeds. Two isoenzymes of aspartate kinase were detected, one sensitive to lysine inhibition and the other sensitive to threonine inhibition. The lysine-sensitive aspartate kinase was predominant, whereas the

threonine-sensitive form appeared to co-purify with the a threonine-sensitive homoserine dehydrogenase, suggesting the existence of a bifunctional threonine-sensitive aspartate kinase/homoserine dehydrogenase as already detected in other plant species. The activities of lysine 2-oxoglutarate reductase and saccharopine dehydrogenase were the lowest observed for any other plant species, including the high-lysine maize mutant *opaque-2*. The presence of a bifunctional lysine 2-oxoglutarate reductase and saccharopine dehydrogenase enzyme was confirmed, however others isoforms were also detected. Considering the activity levels observed for lysine 2-oxoglutarate reductase and saccharopine dehydrogenase in sorghum seeds, the variety used may exhibit a higher level of lysine, which is under investigation. (Financial support from FAPESP, Brazil, and the British Council)

Involvement of amino acids in polyamine synthesis

N. Bagni, P. Accettulli, F. Antognoni, and **A. Tassoni**

Department of Biology e.s., University of Bologna, Via Irnerio 42, 40126, Bologna, Italy

Polyamines are small, positively charged aliphatic amines found in all living organisms. They are involved in a variety of processes in plants, including cell division, organ development, fruit ripening and leaf senescence. The diamine putrescine is mostly produced directly from the amino acid ornithine via ornithine decarboxylase or indirectly from the amino acid arginine via arginine decarboxylase through the intermediate agmatine and N-carbamoylputrescine.

Moreover, there are other sources for putrescine synthesis that were partly neglected in the past years such as for the amino acid citrulline that via the enzyme citrulline decarboxylase represents the only substrate for putrescine synthesis at least in *Sesamum* leaves grown under K^+ deficiency. In sugarcane cell cultures and in activated tissue of *Helianthus tuberosus* tuber, citrulline is the precursor for the additional pathway of putrescine biosynthesis via N-carbamoylputrescine. Recently, a new interest among polyamine researchers was developed toward citrulline, when it was demonstrated that ornithine transcarbamoylase, a very active enzyme involved in the polyamine synthesis, can convert ornithine to arginine through ornithine cycle producing as intermediate compound citrulline.

Citrulline can therefore represent an important source of putrescine via arginine, via ornithine and/or via N-carbamoylputrescine. This compound is also one of the amino acids preferentially translocated through phloem tubes and xylem vessels in Betulaceae and Junglandaceae.

Another amino acid that was recently taken again into consideration in plants as substrate for the synthesis of the diamine cadaverine (1,5-diaminopentane), is lysine. Cadaverine is an important compound synthesised through lysine decarboxylase enzyme in many Leguminoseae, such as soybean seedlings, in Gramineae and Salonaceae where it represents a precursor for alkaloid biosynthesis. In other Leguminoseae, however, like *Lathyrus sativus* seedlings, cadaverine is partly formed from homarginine via homoagmatine through a pathway analogous to that of arginine agmatine and putrescine.

Influence of N-shortage on the amino acid pattern in plants under salinity and heavy metal stress

Y. Friedrich, K. Zirr, R. Mascher, and **H. Bergmann**

Institute of Nutritional Science, Friedrich-Schiller University of Jena, Germany

The area around a former phosphate fertiliser factory is characterised by large concentrations of P, Ca, Na, F and Cd and a low N content. The biochemical behaviour of tree location typical species (*Artemisia vulgaris* L., *Atriplex sagittata* BORKH. and *Elytrigia repens* (L.) DESV.) along a pollution gradient was analysed. Contrary to the in literature (under normal N-levels) described reaction of plants to salt or Cd stress – under N-shortage the total protein content as well as the content of free proteinogeneous amino acids (e.g. proline, glutamine, asparagine) and non proteinogenous amino acids diminished with an increasing pollution of the soil with Na, Ca and heavy metals in the investigation area. With less available N in the soil the plants have to economize on their N-metabolites. So their reaction to the low N-level changes the responses to other chemical stressors.

Regulation of amino acid transporter gene expression by nitrogen and carbon metabolites

Mengjuan Guo[1] and **Daniel R. Bush**[1,2,3]

[1] Physiological and Molecular Plant Biology,
[2] Department of Plant Biology, and
[3] Photosynthesis Research Unit, USDA-ARS, University of Illinois, 190 ERML, Urbana IL 61801, USA

In higher plants, amino acids are the currency of nitrogen exchange between the sites of primary assimilation and the import-dependent tissues. The partitioning of amino acids in this resource allocation process requires the activity of several classes of amino acid transporters in the plasma membrane. The transcript of AAP1, a proton-amino acid symporter, in mature leaf tissue is regulated by nutrient status and environmental cues. After 7 days nitrogen starvation, AAP1 message is highly induced after feeding 25 mM NO_3^- or 10 mM NH_4^+ or 5 mM amino acids such as glutamine, glutamate and asparagine for 30 min. Nitrogen mediated changes in AAP1 message abundance may be due to multiple signals. Inhibitors of nitrate reductase, glutamine synthetase, and aminotransferases do not block induction by NO_3^-, NH_4^+ and asparagine, respectively, while glutamate synthase inhibitors decrease the induction from glutamine, suggesting the importance of the glutamine-glutamate cycle in nitrogen signaling. AAP1 is also induced in dark-adapted plants after 3 hours of illumination. Light dependent changes in expression may be mediated by a specific photoreceptor or by photosynthesis-dependent increases in leaf sugar content. Both 1% sucrose or glucose feeding induces AAP1 message in dark-adapted plants, suggesting light induction might be an indirect effect of sugar-signaling. However, we can not rule out a role for a photoreceptor in regulation of AAP1 message, because far red light illumination decreases the sucrose-dependent induction. Current efforts in the lab include parallel experiments using expression profiling to identify transcription factors associated with changes in AAP1 expression, and developing genetic tools for dissecting this response pathway.

Effect of chromium on proline content and some red – ox enzymes in mentha piperita plants

A. H. Farooqi[1] and **M. Caus**[2]

[1] Central Institute of Medicinal and Aromatic Plants, Lucknow 226015, India
[2] Institute of Plant Physiology, Moldovan Academy of Sciences, Chisinau, MD 2002, Moldova

This investigation was taken up to study the response of M. piperita to Cr impact. M.piperita *cv Tushar* plants were supplied with different CrO_3 concentrations (1 mM, 2 mM and 4 mM). We have examined the reaction of Mentha plants to different CrO_3 concentrations regarding the biomass accumulation, proline content, Nitrate Reductase (NR) activity, isoforms and activity of Peroxidase (PO) and Polyphenoloxidase (PPO), yield and composition of essential oil.

Results indicate that application of the high Cr concentration led to pronounced toxic effect on plant growth, caused decreased in root and leaf fresh weight, brownness, drying and death of upper parts of plant shoots, leaves and even death of whole plants.

The plant growth and development was reduced and new of formed shoots and leaves were much smaller. Proline accumulation enhanced considerably in the leaves under increasing concentrations of Cr. *In vivo* NR activity was inhibited by Cr at all concentrations. The pattern of PO isoforms was modified under high concentrations of CrO_3 treatment. In control and of the low Cr concentration (1 mM) only one isoform of PO was detected. Three new isoforms of PO were expressed at heavy metal stress conditions (2 mM, 4 mM). A positive correlation between the increasing Cr concentrations and intensity of all PO isoforms was observed. The pattern of PPO isoforms was not modified under Cr treatment, but total PPO activity increased. Essential oil yield at 1 mM of CrO3 treatment was not affected. Only some differences in oil composition were evident at this Cr concentration.

Changes in amino acid composition in soybean (*glycine max l.*) in response to titanium treatment

M. Caus and **S. Toma**

Institute of Plant Physiology, Moldovan Academy of Sciences, Chisinau, MD 2002, Moldova

Titanium represents an ultra trace element which is widely spread in the Earth's crust. It takes the 10th place on the frequency list of elements. The role and importance of this ultra trace element in physiological and biochemical processes of plant growth and development was hardly tested during last period and is very controversial. The results of most investigations suggested that titanium is a beneficial element for plants.

This study reports the effect of various concentrations of titanium tetrachloride on the qualitative and quantitative composition of free and bound amino acids (AA) composition in soybean plant organs.

Plants of *Glycine max L.* were grown in greenhouse controlled conditions. Before sowing, soybean seeds, *cv Bucuria* were treated with various concentrations of titanium tetrachloride; 0.015% – Ti 1, 0.030% – Ti 2 and 0.045% – Ti 3. Control seeds were treated with distilled water. The soil was infected with effective nitrogen fixing bacteria *Bradyrhizobium japonicum 9*. Plant samples nodules (N), roots (R) and leaves (L) were collected at the end of plant mass flowering period and fixed in liquid nitrogen and subsequently analyzed for free and bound amino acid compositions.

The results indicate that the treatment of seeds before sowing with Ti leads to changes in free and bound amino acids in soybean organs. Particularly, a decrease is found in the free total AA content in R at all Ti concentrations, while in the control and N – Ti 1 and Ti 2, the value of the total free AA is almost the same. The total free AA increased in L – Ti 2, and decreased in Ti 3. Different effects of various Ti concentrations were observed in the qualitative free AA composition.

The Ti treatment of soybean seeds decreased the sum of the bound AA in N at all concentrations. In R – control and Ti samples pronounced differences were not detected in the total bound AA. In L, Ti 1 and Ti 2 the sum of bound AA increased. It is shown that the influence of Ti on the bound AA composition depends on the plant organ and concentration of Ti.

Regulatory networks of amino acid metabolism in arabidopsis

Gad Galili[1], **Xiaohong Zhu**[1], **Asya Stepansky**[1], **Youli Yao**[1], **Song Luhua**[1], **Shai Ufaz**[2], **Aviah Zilberstein**[2], and **Csaba Konz**[3]

[1] Department of Plant Sciences, The Weizmann Institute of Science, Rehovot, Israel

[2] Department of Plant Sciences, Tel Aviv University, Tel Aviv, Israel

[3] Max-Planck-Institut für Züchtungsforschung, 50829 Köln, Germany

We are interested identifying metabolic networks of amino acid metabolism as well as genes that regulate these networks in response to developmental, physiological, environmental and stress-related signals. Our model system is the pathway of lysine metabolism and its connection with other amino acid metabolic pathways. In these studies, we use transgenic plants expressing a bacterial feedback-insensitive dihydrodipicolinate synthase of lysine biosynthesis, coupled with a knockout mutation in the lysine catabolism pathway. Analysis of these transgenic plants showed that lysine metabolism is concretely regulated both by its synthesis and catabolism and also exposed novel regulatory metabolic networks connecting lysine metabolism with the metabolism of amide amino acids as well as with other amino acids of the aspartate-family pathway. Genetics, genomics, proteomics and metabolic profiling approaches will be used to elucidate genes and mechanisms associated with these novel regulatory metabolic networks.

Biochemical and molecular aspects of lysine oxoglutarate reductase and saccharopine dehydrogenase in rice (*Oriza sativa L.*)

S. A. Gaziola

Departamento de Genética – ESALQ/USP. Piracicaba – SP. CEP13400-970, Brazil

One of the most important deficiency in cereals is the limited content of essential amino acids such as lysine, threonine, tryptofan leading to the low nutricional quality. The essential amino acids lysine is synthetized in higher plants in the aspartic acid biosynthetic pathway, that also leads to the formation of threonine, methionine and isoleucine. Thus, the understanding of the regulation of the metabolism of these amino acids becomes a important task. Two enzymes, lysine 2-oxoglutarate reductase and saccharopine dehydrogenase, which regulate the catabolism of lysine, have been isolated from maize, rice and tobacco. In rice, the LOR and SDH protein was shown to be endosperm-specific. It have been demonstrated in plants that LOR/SDH activities, exist in a single bifunctional protein. Regarding the regulation of the lysine synthesis, there is strong evidence that lysine is also subject toregulation of its catabolism in plants, specifically in the seed. In this work we analyzed different cultivars of rice to determine the specific activity of LOR and SDH. For SDH staining activity in native PAGE and protein expression by Western blotting. Moreover, we isolated a cDNA clone encoding the bifunctional enzyme LOR/SDH from total endosperm RNA by reverse transcription-polymerase chain reactions (RT-PCRs). The results showed that the genotypes presented no diferences in protein expression and in specific activity developed for the 120 kDa band. PAGE staining for SDH activity showed two isoforms of the enzyme. The *LOR/SDH* gene from immature rice seeds of the cultivar IAC-165 showed gene homology with rice (TMRI) and maize.

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The function of sulfur amino acids and their derivatives in the defense of plants against pathogens

Rüdiger Hell and **Ricarda Jost**

Institute of Plant Genetics and Crop Plant Research (IPK), 06466 Gatersleben, Germany

Plants have developed numerous innate and inducible defense mechanisms against bacterial and fungal pathogens. Among the latter are phytoalexins, cell wall degrading enzymes like chitinases and antifungal toxins. Many of these compounds contain reduced sulfur moieties as functional groups that are essentially donated from the amino acids cysteine or methionine. Prime examples are the sulfur-rich peptide classes of the thionins and defensins and the secondary metabolites glucosinolates and the phytoalexin camalexin. Hence, plant sulfur nutrition is assumed to be important to provide reduced sulfur for the formation of these defense compounds. Compatible host-pathogen interactions

often meet less effective defense reactions than incompatible ones, resulting in a variable degree of resistance or tolerance against a pathogen. Therefore, the recent discovery of enhanced tolerance of crop plants with optimal sulfate supply to fungal pathogens provides a new approach to improve plant health and yield.

To this end the functional analysis of such sulfur-induced resistance has been established in an axenic pathosystem consisting of *Arabidopsis thaliana* and either the necrotrophic fungus *Alternaria brassicicola* or the defense signal methyljasmonate under precisely defined nutritional conditions. The experiments aim at the identification of candidate metabolites and genes that co-ordinately respond to pathogen stress and optimal sulfate supply, but not to either condition or sulfate deficiency alone. This approach will lead to the dissection of this phenomenon of quantitative resistance and eventually to the understanding of the underlying mechanisms. Profiling of sulfur metabolites and transcripts indicate that indeed key steps in sulfate reduction, sulfur amino acid biosynthesis and glucosinolate formation are up-regulated in response to induction under the condition that optimal sulfate nutrition is provided.

Current understanding of the regulation of methionine biosynthesis in plants

Holger Hesse[1], **Oliver Kreft**[1], **Michaela Zeh**[1], **Paola Casazza**[2], and **Rainer Höfgen**[1]

[1] Max-Planck-Institut für Molekulare Pflanzenphysiologie, 14424 Potsdam, Germany
[2] University of Milan, Plant Physiology and Biochemistry Section, Biology Department, Via Celoria 26, Milan 20133, Italy

Among the proteinogenic amino acids methionine displays plenty of essential direct or indirect functions in cellular metabolism. Methionine (Met) as a member of the aspartate family plays a major role as protein constituent, in the initiation of mRNA translation and through its main derivative S-adenosylmethionine (SAM). SAM itself has several key functions such as primary methyl-group donor or as precursor for metabolites as ethylene, polyamines, vitamin B1 or 3-dimethylsulfoniopropionate, an osmoprotectant, as well as dimethylsulfide, a source of atmospheric sulfur. Furthermore, Met is an essential amino acid required in the diet of nonruminant animals. Major crops, such as cereals and legumes, are low in Met and an attempt to manipulate the biosynthetic pathway is a major interest of molecular plant breeding. According to this it can be assumed that Met synthesis, accumulation and consumption are under high regulatory control. In plants, O-phosphohomoserine (OPHS) represents the common substrate for both threonine and methionine synthesis. OPHS is either directly converted to threonine by threonine synthase or, in a three step mechanism, to methionine through condensation of cysteine and OPHS to cystathionine, which is subsequently further converted to homocysteine and then methionine by the enzymes cystathionine gamma-synthase, cystathionine beta-lyase, and methionine synthase, respectively. Recent studies suggest that Met synthesis in plants has to be controlled at the level of competition between CgS and TS for their common substrate OPHS. In this paper, a summary of our current understanding of the regulatory network with the focus on efforts to understand and manipulate the carbon flux into Met is given.

Multiparallel analysis of amino acid biosynthesis under sulfur depletion in *Arabidopsis thaliana*

Rainer Hoefgen, Victoria Nikiforova, and **Holger Hesse**

Max-Planck-Institut für Molekulare Pflanzenphysiologie, Department Willmitzer, Am Muehlenberg 1, 14476 Golm, Germany

Sulfate is one of the major inorganic nutrients of plants and sulfur metabolism in plants has gained substantial interest over the past few years. Sulfate assimilation primarily results in the amino acids cystein and methionine, which provide the starting metabolites for a huge number of correlated plant biochemical processes. Through various metabolic routes, sulfur is closely interrelated to a huge number of metabolic processes in plants. Yet, understanding control and regulation of sulfur metabolism, and hence biosynthesis of the amino acids methionine and cysteine is still limited. We approached an in depth analysis of sulfur metabolism through analysing the adaptation of gene expression of *Arabidopsis thaliana* in response to sulfur deprivation using analytical tools such as array hybridisations and metabolite profiling. *Arabidopsis* seedlings were grown under different time regimes on sulfur depleted media and gene expression patterns were analysed using array hybridisation. Special efforts have been made to design the experiment in a way to obtain statistically valid data. The resulting RNA was used to challenge the MSU collection of 16,000 ESTs spotted on nylon filters or, recently, full genome affymetrix chips. Analysis was performed using the MPI-MP *Haruspex* database and various bioinformatical tools. A whole network of genes was identified to respond to sulfur starvation, among them as expected genes of various pathways. Correlation analysis elucidated co-ordinated interaction of these pathways finally triggering physiological responses. Examples of this as acting on amino acid biosynthesis will be presented as well as approaches to sort the flood of data using bioinformatic tools. The long term goal will be an integrated description of the signaling and control mechanisms of sulfur amino acid metabolism in plants, initially using *Arabidopsis* as a model.

Structure/biological function relationship studies on the plant peptide hormone phytosulphokine-α (PSK-α)

Agata Bahyrycz[1], **Yoshikatsu Matsubayashi**[2], **Mari Ogawa**[2], **Youji Sakagami**[2], and **Danuta Konopinska**[1]

[1] Faculty of Chemistry, University of Wroclaw, 50-383 Wroclaw, ul. F. Joliot-Curie 14, Poland
[2] Graduate School of Bio-Agricultural Sciences, Nagoya University, Chikusa, Nagoya 464-8601, Japan

Phytosulphokine-α (PSK-α) a sulfated growth factor (H-Tyr(4-OSO_3H)-Ile-Tyr(4-OSO_3H)-Thr-Gln-OH) universally found in both monocotyledons and dicotyledons, strongly promotes proliferation of plant cells in culture. In our studies on structure/activity relationship in PSK-α we performed the synthesis of series analogues, such as: [H-D-Tyr(4-OSO_3H)^1] – (9), [H-Phe(4-SO_3H)^1] – (10), [H-D-Phe(4-SO_3H)^1] – (11), [H-Phg(4-SO_3H)^1] – (12), [H-Phg(4-SO_3H)^1] – (13), H-Phe(4-NH-SO_2CH_3)^1] – (14), [H-D-Phe(4-NH-SO_2CH_3)^1] – (15), [H-Phe(4-NO_2)^1] – (16), [H-D-Phe(4-NO_2)^1 – (17), [H-Phg(4-NO_2)^1] – (18), [H-D-Phg(4-NO_2)^1] – (19), [H-Hpa(4-NO_2)^1] – (20), [H-Phg(4-OSO_3H)^1] – (21), [Phe(4-NO_2)^3] – (22), [Phg(4-NO_2)^3] – (23), [Hpa(4-NO_2)^3] – (24), [H-Phe(4-SO_3H)^1, Phe(4-SO_3H)^3] – (25) [H-Phe(4-NO_2)^1, Phe(4-NO_2)^3] – (26), [H-Phg(4-NO_2)^1, Phg(4-NO_2)^3] – (27), [H-Hpa(4-NO_2)^1, Hpa(4-NO_2)^3] – (28) and [Val^3] – PSK-α (29). For modification of the PSK-α peptide chain the novel amino acids and their derivatives were synthesized such as: L-Phg(4-SO_3H) (**1**), D-Phg(4-SO_3H) (**2**), Fmoc-Phg(4-SO_3H) (**3**), Fmoc-D-Phg(4-SO_3H) (**4**), Boc-Phg(4-$NHSO_2CH_3$) (**5**), Boc-D-Phg(4-$NHSO_2CH_3$) (**6**), Boc-Phe(4-$NHSO_2CH_3$) (**7**), and Boc-Phe(4-$NHSO_2CH_3$) (**8**). Peptides were synthesized by solid phase method according to Fmoc-procedure on Wang-resin. Free peptides were released from resin by 95% of TFA in presence of EDT. All peptides were tested by competitive binding assay to the carrot membrane using ^{3}H-labeled PSK according to the Matsubayashi et al., 2002 test.

Can photorespiratory amino acid metabolism be short-circuited?

J. F. C. Carvalho[1], **M. A. J. Parry**[1], **P. J. Madgwick**[1], **A. J. Keys**[1], **M. Beale**[1], **J. L. Ward**[1], and **P. J. Lea**[2]

[1] Rothamsted Research, Harpenden, AL5 2JQ, UK

[2] Biological Sciences, Lancaster University, Lancaster. LA1 4YQ, UK

Transgenic tobacco (*Nicotiana tabacum*) plants have been generated in which the part of the photorespiratory cycle that converts glycine to serine has been bypassed. The plants have been transformed with the genes *gcl* and *hyi* encoding respectively two enzymes isolated from the bacterium *Escherichia coli*, glyoxylate carboligase (gcl; EC 4.1.1.47), which converts glyoxylate to tartronate semialdehyde and CO_2, and hydroxypyruvate isomerase (hyi: EC 5.3.1.22), which converts tartronate semialdehyde to hydroxypyruvate. The two enzymes should short circuit photorespiratory metabolism and avoid the decarboxylation of glycine and the generation of ammonia. The new pathway should decrease the energy requirements of C_3 photosynthesis, by avoiding the necessity of reassimilating the NH_3 released in the mitochondrial reactions. The transgenic lines 32, 33 and 37 expressing the *E. coli* gene for *gcl* modified by the addition of a peroxisome targeting sequence and the lines 79, 84 and 92 bearing both *gcl* and *hyi* transgenes appeared to grow normally under low light or elevated CO_2 concentrations. Under photorespiratory conditions, less ^{14}C-glycolate was metabolised to glycine and serine and more to sucrose in the transgenic line than in the wild type plants. The amounts of glutathione in the leaves were variable in *gcl* and *gcl-hyi* lines but were always greater than the wild type. Amino acid quantification revealed that the glutamine: glutamate and the glycine:serine ratios were altered in the *gcl* and *gcl-hyi* lines. ^{1}H-NMR has been used to further examine differences between null and *gcl* lines growing at high CO_2 and in the glasshouse.

A systems based approach to C:N signaling in plants

Laurence Lejay[1], **Andrei Kouranov**[1], **Peter Palenchar**[1], **Alexis Cruikshank**[1], **Michael Chou**[1], **Dennis Shasha**[2], and **Gloria Coruzzi**[1]

[1] New York University, Department of Biology, 100 Washington Square East NY, NY 10003, and

[2] Courant Institute of Mathematical Sciences, Department of Computer Science, New York University, 251 Mercer St. NY NY 10012, USA

Studies have shown that multiple input signals can affect expression of amino acid biosynthesis genes in plants. We were specifically interested in determining how multiple input signals such as light, carbon and nitrogen intersect to regulate genes involved in nitrogen assimilation using Arabidopsis as a model. We initially considered six binary input signals; light, carbon, inorganic N, glutamate, glutamine and starvation. Examining all possible combinations of these six inputs would result in 64 treatments (2^6). To design a small set of experiments that would systematically sample and effectively cover this experimental space we employed a math tool, initially developed for software testing, called Combinatorial Design (CD). We validated this CD approach by comparing CD experiments to a complete dataset of 64 treatments and we performed Boolean analysis in order to model circuits for regulation of gene expression by multiple input signals. We are also performing gene chip experiments on CD samples to monitor the regulation of all amino acid biosynthesis genes by these multiple input signals. For this analysis, we developed a bioinformatic tool, called "PathExplore" that can be used to query microarray expression datasets to determine how all the genes in pathways are regulated. The "PathExplore" database includes genes for biosynthetic pathways of N-assimilation, all amino acid biosynthesis pathways and related co-factors, as well as C-metabolism pathways, and signaling pathways. Combinatorial Design plus gene chip analysis with "PathExplore" should enable us to model genome wide regulatory circuits, a first step to the construction of a virtual plant.

The role of competition between cystathionine gamma-synthase and threonine synthase in controlling the synthesis of methionine and threonine in *Arabidopsis thaliana*

Thomas Leustek

Rutgers University, Biotechnology Center for Agriculture and the Environment, 59 Dudley Road, New Brunswick, New Jersey, 08901-8520, USA

Homoserine kinase (HSK) produces *O*-phospho-L-homoserine (OPH), the substrate for cystathionine -synthase (CGS), the first enzyme for Met synthesis, and threonine synthase (TS), the first enzyme for Thr synthesis. To test the role that competition for OPH has on Met and Thr synthesis, transgenic *Arabidopsis thaliana* were prepared that overexpress HSK, CGS, or *Escherichia coli* TS (eTS) under transcriptional control of the CaMV 35*S* promoter. HSK overexpression caused OPH, Met and Thr to accumulate only when plants were fed homoserine. During homoserine feeding for periods up to 72 hours the HSK-overexpressing plants produced much more OPH than wild type, but Met and Thr accumulation was similar to wild type. After homoserine feeding, 9 to 21 times more Thr accumulated than did Met. Overexpression of CGS caused Met to accumulate, but did not affect Thr level. eTS expression caused Thr to accumulate, but did not affect Met level. When the CGS-overexpressing plants were fed homoserine, Met accumulated more rapidly and OPH and Thr accumulation was reduced compared with wild type plants. When eTS-expressing plants were fed homoserine, Thr did not accumulate at a rate significantly greater than wild type, but OPH and Met accumulation were reduced compared with wild type. These results support the idea that partitioning of OPH between Met and Thr depends upon the relative activities of CGS and TS. The fact that under normal growth conditions neither CGS or eTS overexpression reduces the level of the amino acid from the competing pathway suggests that the rate of OPH synthesis may increase in response to increased OPH utilization.

Effects of arsenate stress on antioxidative enzymes, glutathione and polyamines in higher plants

R. Mascher, B. Lippmann, S. Holzinger, A. Neagoe, W. Scheiding, B. Knoch, and **B. Bergmann**

Institute of Nutritional Science, Friedrich-Schiller University of Jena, Germany

Arsenic (As) is a ubiquitous toxic element in environment. Arsenate and arsenite are the primary chemical forms occuring in soils. The concentration tolerated by plants varies from 1 to 50 mg arsenic per kg soil.

We have investigated the antioxidant responses of red clover plants (*Trifolium pratense*) and mustard (*Sinapis alba*) to arsenate treatment. Analyses of long time stress response, clover plants were grown for 10 weeks in a greenhouse amended with different concentrations of Na_2HAsO_4 (40, 80 and 400 μM As in soil) or a heavy metal mixture (40 μM Cd, 4 mM Zn and 80 μM As in soil), respectively. The analyses of short time stress response, seedlings were grown in increasing concentrations of Na_2HAsO_4, ranging from 40–160 μM As, for up to 7 d in a hydroponic *in vitro* system.

Roots and shoots were analysed for antioxidative enzymes and molecules. Increases in superoxide dismutase (SOD) activity, peroxidase activity as well as decreases in chlorophyll and carotenoid concentrations were correlated with increasing arsenic content in plants. The analyses of native PAGE SOD activity staining indicated one Mn-SOD and two major Cu/Zn superoxide dismutase isoenzymes in clover shoots, whose activity increased in response to arsenate treatment. Glutathione content was reduced at the highest As concentration applied, this indicate a strong synthesis of phytochelatins for detoxification process.

The concentrations of the polyamines spermine/spermidine and the diamine putrescine in the shoots of clover plants were increased at the 80 μM As application in soil. At the 400 μM soil treatment, the concentrations of spermine and spermidine decreased and those of the diamine putrescine further increased. Thus we detected a significantly increased putrescine content at 80 and 400 μM concentration in soil, putrescine increasing by about 84% at the highest arsenate soil content. 400 μM As in soil induced the highest spermine accumulation in the shoots (raising to about 263%). This could be seen as a protective mechanism against the destruction of membranes caused by arsenate stress.

Antioxidative polyamines (PAs) were accumulated in plants at higher doses of arsenate in the soil. Increase in SOD activity and accumulation of PAs as well as chlorophyll loss could be prevented by application of Zn and Cd together with As. The results indicate that higher doses of arsenate produce oxidative damage in clover shoots. The protective role of antioxidative enzymes, glutathione and PAs will be shown.

Levels of endogenous free amino acids during induction phase of shoot organogenesis in leaves of pineapple cultured *in vitro*

Adélia Y. Kitakawa, Regina M. Hamasaki, and **Helenice Mercier**

Department of Botany, São Paulo University,
CP 11461 CEP 05422-970, São Paulo, SP, Brazil

A wide range of culture conditions influences growth and regeneration *in vitro*. The usual strategy to start adventitious organogenesis in pineapple (*Ananas comosus* var Smooth Cayenne) is to culture leaf explants in Knudson medium containing NAA ($1.0\,mg\cdot l^{-1}$) and BA ($2.0\,mg\cdot l^{-1}$), which results in the formation of protuberances and their further development into shoots and plantlets. The highest percentage of cultivated leaves forming protuberances is 40%. However, when the medium was supplemented with glutamine (8 mM) there was approximately two-fold increase in that percentage. Also, a more vigorous shoot growth was observed in the presence of glutamine. There is little information on the mechanisms by which amino acids may interact with growth regulators to ensure high rates of organogenesis. It has been proposed that the specific levels of endogenous free amino acids are important in the regulation of plant growth. In the present study, it was found that the endogenous levels of glutamine and asparagine varied during the induction phase. The contents of these amino acids increased markedly in the first 36–72 hs of culture. The control (glutamine-free medium) did not result in an increase of endogenous glutamine and asparagine. These observations suggest that the endogenous glutamine and asparagine contents in pineapple leaf explants incubated in glutamine-free medium are possibly limiting signals associated with induction of shoot organogenesis.

Identification of cystine lyase gene in *Arabidopsis thaliana*

Patrik Jones and **Kazuki Saito**

Graduate School of Pharmaceutical Sciences, Chiba University,
CREST of Japan Science and Technology Corporation (JST),
Yayoi-cho 1-33, Inage-ku, Chiba 263-8522, Japan

Cystine lyases catalyze the breakdown of L-cystine to thiocysteine, pyruvate and ammonia. Until now there are no reports of the identification of a plant cystine lyase at a molecular level and it is not clear what biological role this class of enzymes have in plants. Based on the amino acid sequence of cystine lyase from *Brassica oleracea*, we identified candidate genes encoding cystine lyase in *Arabidopsis thaliana* showing highest homology to the deduced amino acid sequences annotated as tyrosine aminotransferase-like, including a coronatine, jasmonic acid and salt stress inducible gene, CORI3 (78.8% identity), and the unidentified rooty/superroot1 gene (44.8% identity). A full-length EST clone of CORI3 was obtained and recombinant CORI3 was synthesized in *Escherichia coli*. Isolated recombinant CORI3 catalyzed a cystine lyase reaction, but no aminotransferase reactions. The present study identifies, for the first time, a cystine lyase from plants at a molecular level and redefines the functional assignment of the only functionally identified member of a group of *A. thaliana* genes annotated as tyrosine aminotransferase-like.

Amino acids in cereals under heavy metal ions exposure

O. O. Sorochan and **N. I. Shtemenko**

Dniepropetrovsk National University, Dniepropetrovsk 49050, Ukraine

It was supposed that amino acids being secreted into risosphere play role of phytosiderophores. We showed that some lines and hybrids of cultural cereals (Zea mays L.) and wild cereals (Festuca rubra L., Lollium perenne L.) had different quantity and content of amino acids in root exudates. Application of some growth regulators were elaborated. We studied the process of exudation of amino acids by roots of the species during growing on sterile sand. Growth stimulators increased total quantity of amino acids in root exudates in 1,5–2 times. The abundant amino acids were Glu, Asp and their amides. In the next part of experiments during growing on Pb, Zn and Cd soils in the cases of growth stimulation we showed that free amino acids of green parts of the plants differed from control ones and were characterized by high variability. Total quantity of free amino acids in primary leaves of experimental plants increased approximately in 5–12 times. Especially high was quantity of free Asp and aromatic amino acids. Accumulation of Asp was especially sharp and common for tissues of the studied wild cereals. Asp is a multifunctional amino acid and its accumulation under influence both toxic metal and the growth regulator may be explained by its transport function: in the process of adaptation it was necessary to support reparative processes for which transport of metabolites is necessary; growth stimulator introduction led to acceleration of oxaloacetate-aspartate transamination pathway; as a multifunctional amino acid it could take part in different biosynthetic processes.

Changes in protein and amino acid content of *Brassica juncea* due to fungal infection

Manju Tomar

Environmental Biology Laboratory, Department of Botany,
University of Delhi, Delhi-110007, India

Mustard, *Brassica juncea* (Linn.) Czern. and Coss. is an annual oilseed crop grown in different parts of India. The crop is grown in October–November and the seeds harvested in March–April. The seeds are stored through summer and monsoon in warehouses in gunny bags. Before storage, the seeds are not subjected to any protective treatment. Associated with storage of seeds in warm and humid climate is the problem of fungal infection.

An assessment was carried out to study the amino acids, protein content and subcellular changes in the protein bodies of fungal infected seeds in comparison to healthy seeds. Significant changes in the quantity of amino acids were recorded whereas depletion was observed in the protein contents of fungal infected seeds.

Ultrastructural studies revealed that the cell of healthy seeds has a large nucleus, numerous protein bodies and a profusion of compactly arranged lipid bodies distributed uniformly in the cytoplasm. Protein bodies were spherical, some appeared to be homogenous while others contained globoid inclusions. In fungal infected seeds during degradation of protein bodies some changes were noticed in their shape and electron density. The early changes involved considerable swelling of protein bodies and their inclusions. Later the protein bodies fused and formed prominent masses of loose protein material. Finally, the discrete protein bodies disappeared and were gradually replaced by vacuoles. Degradation of protein bodies was followed successively by an increase

in intensity of cytoplasm staining and proliferation of endoplasmic reticulum and organelles such as dictyosomes and mitocondria.

Engineering of essential amino acids in soybean for animal feed

Tim Ulmasov[1], **Bill Rapp**[1], **Lisa Weaver**[1], **Qi Wang**[1], **Gabriela Vaduva**[1], **Laura Crow**[1], **Joel Ream**[1], **Betsy Hill**[1], **Wayne Brown**[1], **Steve Kan**[1], **Jihong Liang**[2], and **Ken Gruys**[1]

[1] Monsanto Company, 800 N. Lindbergh Blvd., St. Louis, MO 63167, USA

[2] Renessen LLC, Suite 300 South 3000 Lakeside Drive, Bannockburn, IL 60015, USA

Soybeans represent a valuable source of protein for both human and animal nutrition. Although present in large amounts, the protein in soybeans does not contain a balanced proportion of dietary essential amino acids. As part of an effort to create soybeans with more favorable amino acid compositions, we are developing technologies to generate transgenic soybeans that are enriched in selected essential amino acids. Two approaches are being used to increase the levels of amino acids. One involves modifying a particular biosynthetic pathway to cause an accumulation of free amino acid in the seed. The second approach is relying on the knowledge of crystal structure and computer modeling technology to modify existing soybean seed storage proteins to improve their essential amino acid composition, and expressing such modified proteins at high levels in soybean endosperm. A combination of these approaches should improve the amino acid composition of the soybean, and increase its value as a source of protein to meet human dietary needs and for livestock feed formulations.

Polyamines

Identification of polyamine modulons and regulation of gene expression by the modulons

M. Yoshida[1], **K. Kashiwagi**[1], **A. Ishihama**[2], and **K. Igarashi**[1]

[1] Graduate School of Pharmaceutical Sciences, Chiba University, Chiba, [2] Division of Molecular Biology, Nippon Institute of Biological Sciences, Tokyo, Japan

We proposed that genes whose expression is modulated by polyamines at the level of translation are referred to as a ''polyamine modulon''. Those were *oppA*, *cya* and *rpoS* genes. The mechanism of polyamine stimulation was as follows: polyamines cause a structural change of the SD (Shine-Dalgarno) sequence and the initiation codon AUG of OppA mRNA, facilitating formation of the initiation complex; polyamines stimulate interaction between the initiation codon UUG of Cya mRNA and the anticodon CAU of fMet-tRNA$_i$; and polyamines stimulate the readthrough of the amber codon of RpoS mRNA through its stimulation of Gln-tRNA^{supE} binding to ribosomes. In this study, we identified *fecI*, *fis* and *iclR* genes as part of a polyamine modulon. Synthesis of FecI, Fis and IclR proteins was stimulated by polyamines, and the SD sequences of these mRNAs were not obvious or not located at the usual position on mRNA. When the SD sequences were created at the normal position on these mRNAs, synthesis of these proteins was no longer influenced by polyamines, although basal synthetic activity of proteins greatly increased. These three polyamine modulons stimulate gene expression of mRNAs whch are involved in iron uptake and the production of ATP. Polyamine modulons thus far found were mainly related to the expression of mRNA. By DNA microarray, we found 309 genes were upregulated by polyamines among 2742 genes. Those genes are probably involved in polyamine modulation of cell proliferation.

Polyamine catabolism

Nikolaus Seiler

INSERM U 392, Institut de Recherche contre les Cancers de l'Appareil Digestif (IRCAD):1, place de l'hôpital, Bp. 426, 67091 Strasbourg Cedex, France

Owing to the establishment of cells and transgenic animals which either lack or overexpress, acetylCoA:spermidine N^1-acetyltransferase (SAT), a major progress was made in our understanding of the role of SAT. Cloning of polyamine oxidases of mammalian cell origin revealed the existence of several enzymes with different properties. One appears to be identical with the polyamine oxidase that was postulated to catalyse the conversion of spermidine and spermine to putrescine within the interconversion cycle. The other oxidases are presumably spermine oxidases, because they prefer free spermine to its acetyl derivative as substrate. Transgenic mice and cells which lack spermine synthase revealed that spermine is not of vital importance for the mammalian organism, but its transformation into spermidine is nevertheless a vitally important reaction.

Numerous metabolites of putrescine, spermidine and spermine are known as normal constituents of cells, and more frequently as urinary excretory products, which are presumably the result of diamine oxidase-catalysed oxidative deaminations. The recent methods of diamine oxidase purification will allow one studying substrate properties of different intermediates. The fact that spermine in contrast with spermidine is a poor substrate of purified diamine oxidase, but is readily transformed into N^8-(2-carboxyethyl)spermidine *in vivo* will need clarification. There were numerous attempts to establish diamine oxidase as a regulatory enzyme of polyamine metabolism. Based on the slow turnover of diamine oxidase, the antiproliferative effect of 4-aminobutyric acid, a major product of putrescine catabolism, and the efficacy of polyamine regulation by the interconversion pathway, a defence mechanism of DAO appears more likely than a regulatory function.

In vitro chemosensitivity tests for cancer patients: the use of ornithine decarboxylase

U. Bachrach

Department of Molecular Biology, Hebrew University-Hadassah Medical School, Jerusalem, Israel

Despite the progress made in cancer chemotherapy and the introduction of the so called ''protocols'' for treating patients, the outcome of treatments did not meet the expectations. Unfortunately, individual patients with apparently identical tumor biologies do not always respond identically to the same drug regimen. We have recently developed a new *in vitro* chemosensitivity assay to predict the potential response of hematological cancer patients to therapy. This approach is based on the well known fact that ornithine decarboxylase can be defined as an early marker of proliferation. This marker was detected in drug-treated lymphocytes, by quantitative immuno-histological analyses, using an ODC antibody and a FITC-conjugated goat anti-rabbit second antibody. Slides were finally examined with a confocal laser microscope, Phoibos 1000. Drug resistance was detected in five patients who subsequently deceased. Lymphocytes from normal individuals were sensitive to all drugs tested, whereas 40 leukemia and lymphoma patients showed different sensitivities to certain drugs. Bone marrow from cancer patients was also tested, using 100–1,000 cells per assay, which was completed

within 2 days. The test also permitted testing of new drugs on the proliferation of lymphocytes from hematological cancer patients. Recently, we simplified the test by developing an ELISA assay to detect ornithine decarboxylase. This modification of the test gave promising results. Studies are now in progress to test specimens from solid tumors. We hope that this new methodology will improve anti-cancer treatment, will safe lives and reduce unnecessary harmful side effects.

Polyamine-responsive regulation of the polyamine biosynthetic pathway by mRNA translational mechanisms

Anthony J. Michael, Marina Franceschetti, Colin Hanfrey, Melinda Mayer, Katherine Elliott, Crista Illingworth, Mark Collier, Benjamin Thompson, and **Barry Perry**

Institute of Food Research, Norwich Research Park, Colney, Norwich, NR4 7UA, UK

The polyamines spermidine and spermine are essential for growth and cell proliferation in most organisms. Modification of polyamine metabolism results in changes to mRNA translation and ribosome function prior to changes in transcription and DNA replication. Polyamines influence the production of the ornithine decarboxylase antizyme by modifying ribosomal frame-shifting efficiency on the antizyme mRNA. The mammalian S-adenosylmethionine decarboxylase (AdoMetDC) is regulated in response to polyamine levels by interaction of polyamines with a ribosome stalling nascent peptide encoded by a sequence-dependent upstream open reading frame. We have shown that the plant AdoMetDC mRNA is translationally regulated by polyamines through a novel mechanism involving ribosomal leaky scanning. It is our hypothesis that the polyamine biosynthetic pathway in diverse organisms is regulated in response to polyamines by translational mechanisms because translation is particularly sensitive to changes in polyamine levels. The translational mechanisms employed in regulating the polyamine pathway have evolved to be more sensitive to polyamines than is cellular translation in general. Thus these translational mechanisms can be thought of as early warning systems to detect polyamine-mediated changes to translation and to respond by repressing translation of the polyamine biosynthetic enzymes.

Cellular effects of polyamine analogues in human leukaemic cells

Alison V. Fraser[1], **Patrick M. Woster**[2], and **Heather M. Wallace**[1]

[1] Department of Medicine and Therapeutics and Biomedical Sciences, University of Aberdeen, UK

[2] Department of Pharmaceutical Sciences, Wayne State University, Detroit Michigan, USA

Polyamines have been shown to be necessary for cell growth and differentiation, and this is emphasised in tumour cells where both increased polyamine concentrations and enhanced rates of proliferation are found. Modulation of intracellular polyamine content *in vitro* through the use of single enzyme inhibitors of polyamine biosynthesis has been shown to both deplete polyamine content and induce growth arrest. However, the failure of polyamine enzyme inhibitors *in vivo* has led to the development of a new class of compound, the polyamine analogues. The analogues are related to the natural polyamines structurally, and regulate polyamine homeostasis but do not substitute for them in terms of function. Our studies have used a human leukaemic cell model to assess the chemotherapeutic potential of two of the unsymmetrically substituted polyamine analogues, CHENSpm and IPENSpm. These analogues were both cytotoxic effectively over 48 h. The analogues accumulated within the cell and resulted in depletion of the natural polyamine pools. Unlike the single enzyme inhibitors such as DFMO that induced a state of cellular stasis, treatment with these analogues resulted in cell death through an apoptotic pathway. The pathway to apoptosis was shown to be through release of mitochondrial cytochrome *c*, the so-called stress-induced pathway. Studies with normal cells showed these analogues to be selectively cytotoxic to tumour cells, suggesting they may be useful agents in future chemotherapeutic strategies.

Spermine enzymatic oxidation products cause cytotoxicity in multidrug resistant human melanoma cells overexpressing P-glycoprotein

Enzo Agostinelli[1], **Fabrizio Martelli**[1], **Marianna Palumbo**[1], **Valentina Giovannini**[1], **Giuseppe Arancia**[2], **Laura Toccaceli**[2], **Manuela Marra**[2], and **Agnese Molinari**[2]

[1] Department of Biochemical Sciences "A. Rossi Fanelli", University of Rome "La Sapienza" and CNR, Centre of Molecular Biology, Piazzale A. Moro 5, 00185 Rome, Italy

[2] Ultrastructures Laboratory, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy

The occurrence of resistance to cytotoxic agents in tumor cells is one of the major obstacle to successful anticancer chemotherapy. Multidrug resistance (MDR) is associated with several phenotypic alterations. Cells with the MDR phenotype display decreased drug accumulation due to overexpression of P-glycoprotein (P-gp), which acts as an energy-dependent pump involved in extrusion of drugs. Taking in account our previous studies on human adenocarcinoma cells, the cytotoxic effect induced by bovine serum amine oxidase (BSAO) and spermine was also examined, either in human drug-sensitive (M14 WT) or drug-resistant (M14 DX) melanoma cells. The purpose of this research is to suggest a new strategy to overcome MDR of human cancer cells by using BSAO, which generates cytotoxic products from spermine, H_2O_2 and aldehyde(s). The cytotoxicity induced by BSAO and spermine appears to be imputable both to H_2O_2 and aldehydes produced by the enzyme. Cell survival experiments showed that M14 DX were more sensitive than M14 WT cells, at 37°C. Cytotoxicity was partially inhibited by catalase, suggesting that enzymatically generated H_2O_2 was in part responsible for the cytotoxic effect. The gradual decrease in the percentage cell survival in the presence of catalase at later times suggested that other oxidation products, such as aldehyde(s) or its breakdown product acrolein, could also contribute to cytotoxicity. The cytotoxic effect observed in M14 cells was dependent on the exposure time and spermine concentration. The results obtained on both human cell lines might be of great interest and suggest that the products formed from spermine and BSAO could be used in anticancer therapy, mainly against multidrug resistant tumor cells.

Enzymotherapy of murine melanoma B16 by direct intratumoral injection of native or immobilized bovine serum amine oxidase

Guy Fortier[1], **Enzo Agostinelli**[2], **Anissa Chérif**[1], and **Diana A. Averill-Bates**[1]

[1] Département de Chimie et Biochimie, Université du Québec à Montréal, C.P. 8888, Succursale Centre-Ville, Montréal (Québec) H3C 3P8, Canada

[2] Department of Biochemical Sciences (A. Rossi Fanelli), University of Rome 'La Sapienza' and CNR Centre of Molecular Biology, Piazzale Aldo Moro 5, 00185 Roma, Italy

Considerable interest has been devoted to the role of polyamines (putrescine, spermine and spermidine) in cell development and regulation of rapidly growing tissues such as neoplastic tissues. It was shown *in vitro* that amine oxidase from bovine serum (BSAO, EC 1.4.3.6, diamine oxidase) catalyzed the transformation of spermine into cytotoxic products (hydrogen peroxide and acrolein) which induced cell death.

In this collaborative study, we have investigated the potential of amine oxidase for the *in vivo* treatment of a localized solid tumor. We believe that, *in situ*, the combination of the generation of cytotoxic products and the local depletion of polyamines, will induce a marked change in the growth rate of the tumor. In this study, the C57BL mouse and its syngenic melanoma B16, a non disseminating tumor, were used. The level of polyamines in the blood of C57BL mice, with or with out tumor cells implanted subcutaneously in their lower back, was assessed by pre-column fluorescent derivatization and quantified using reversed phase HPLC. Bovine serum amine oxidase used herein was electrophoretically homogeneous. Immobilization of the enzyme was performed by cross-linking the enzyme during the synthesis of a hydrogel composed of serum albumin and poly(ethylene glycol). The immobilization procedure did not impair enzymatic activity and a good yield of immobilization was obtained. Mice were injected with B16 melanoma cells and tumors were grown until they reached 0.02 g or 0.07 g. Then, mice were divided into various treatment groups, which received saline solution, hydrogel alone, or various doses of native BSAO or immobilized BSAO preparations, injected directly into the tumor. Along with the results of the effects on tumor growth, we will also discuss the mechanism of death cell (necrosis or apoptosis) involved following the various treatments.

Induction of apoptosis by exogenous spermine and amine oxidase in mouse melanoma cells

Diana A. Averill-Bates[1], **Enzo Agostinelli**[2], **Qixiang Ke**[1], and **Guy Fortier**[1]

[1] Département de chimie et biochimie, Université du Québec Montréal, CP 8888, Succ. Centre Ville, Montréal, Québec, Canada H3C 3P8
[2] Department of Biochemical Sciences (A. Rossi Fanelli), University of Rome ''La Sapienza'' and CNR Centre of Molecular Biology, Rome, Italy

Bovine serum amine oxidase (BSAO) is a copper enzyme, which oxidatively deaminates polyamines containing primary amine groups, such as spermine and spermidine. The reaction products are H_2O_2, ammonia and aldehydes such as acrolein. Targeting polyamines has emerged as a promising therapeutic strategy since these ubiquitous polycations play an important role in the development and maintenance of neoplastic growth. Moreover, rapidly growing tissues such as tumors have elevated levels of polyamines due to enhanced putrescine synthesis by ornithine decarboxylase and increased uptake of polyamines. To take advantage of this differential effect between normal and tumor cells, toxic products such as H_2O_2 and aldehyde(s) could be generated *in situ* by amine oxidases for the selective killing of tumor cells. The purpose of this study is to determine whether oxidation products of spermine cause cell death by apoptosis or necrosis in B16 mouse melanoma cells. Cell death by necrosis leads to membrane damage and liberation of cell contents into surrounding tissues, leading to inflammation. Apoptosis is a highly regulated process which ultimately leads to DNA fragmentation and formation of cellular fragments, which avoids damage to neighbouring tissue. BSAO and spermine caused cell death by both apoptosis and necrosis. Apoptosis involved activation of caspase enzymes. Cell death was decreased by both catalase and aldehyde dehydrogenase, which remove H_2O_2 and aldehyde(s), respectively. In conclusion, BSAO could prove to be useful in cancer treatment.

Getting Ras transformed cells to grow old

Joseph Satriano

Department of Medicine, Univ. of California, San Diego and VASDHS, San Diego, CA, USA

Senescence is a tumor suppressive mechanism. This barrier must be overcome for cells to proliferate indefinitely. Agmatine is an endogenous biogenic amine unique in its capacity to induce antizyme and suppress intracellular polyamine levels required for proliferation. We and others have shown that agmatine enters mammalian cells via the polyamine transporter. As polyamine transport is positively correlated with proliferation rate, we examined the effects of agmatine on several mammalian cell lines. We demonstrate an increased sensitivity of transformed cell lines, relative to their non-transformed counter-parts, to the antiproliferative effects of agmatine due, in part, to preferential import and accumulation. This decreased growth response is attributed to an active G1 arrest of the cell cycle, and is independent from apoptotic attrition. In Ras/3T3 cells treated with agmatine we observe a temporal increase in antizyme expression. In this temporal profile (1–10 days) we also observe an early transient induction of the cyclin kinase inhibitor p21Cip1 and a later, sustained expression of p16INK4A, coincident with the repression of cyclin D expression, Rb hyperphosphorylation and cyclin A expression. Furthermore, Ras/3T3 cells administered agmatine demonstrate a loss of response to mitogenic stimuli, changes in morphology and induction of senescence associated B-galactosidase activity, all indicative of senescence. All together, these data demonstrate a reversion from a transformed to a senescent phenotype and support agmatine as a potential tumor suppressor molecule.

Putative ODC activity in *Arabidopsis thaliana*: inhibition and intracellular localization

Annalisa Tassoni, Silvia Fornalè, and **Nello Bagni**

Department of Biology e.s., University of Bologna, Via Irnerio 42, 40126, Bologna, Italy

Ornithine decarboxylase is one of the enzymes involved in the synthesis of putrescine is produced directly from ornithine via ornithine decarboxylase enzyme.

In this work we studied putative ornithine decarboxylase activity in leaves of *Arabidopsis thaliana* L. (ecotype Columbia) plants at non-flowering stage (about 21 days of culture). Putative ODC activity was higher in the particulate than in the soluble fraction and activity was pH-dependent, increasing linearly with the pH. Inclusion of 10 mM arginine in the assay showed that the incidence of ornithine transcarbamoylase activity accounted for about 35% in the particulate fraction, but that its contribution was negligible in the soluble fraction. Increasing concentrations of the irreversible inhibitor α-difluoromethylornithine (DFMO) progressively inhibited putative ODC activity with a 40% inhibition at 20 mM DFMO. Taking into consideration the incidence of ornithine transcarbamoylase activity, the total inhibition of putative ODC activity was of about 75%. Fractionation experiments permitted measurement of putative ODC activity in the nuclei- and chloroplast-enriched fractions. The assays performed on membranes and stromal fractions isolated from gradient purified chloroplasts showed that the enzyme activity was associated almost totally with the plastid membranes.

Bovine serum amine oxidase and spermine induce mitochondrial modifications on multidrug resistant human colon adenocarcinoma cells

Giuseppe Arancia[1], **Annarica Calcabrini**[1], **Manuela Marra**[1], **Pasqualina Crateri**[1], **Alessandro Martone**[2], **Laura Dalla Vedova**[2], and **Enzo Agostinelli**[2]

[1] Ultrastructures Laboratory, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, and
[2] Department of Biochemical Sciences ''A. Rossi Fanelli'', University of Rome ''La Sapienza'' and CNR, Centre of Molecular Biology, Piazzale A. Moro 5, 00185 Rome, Italy

This study explores the possibility of using an enzyme able to generate cytotoxic products from polyamines present in tumor cells. H_2O_2

and aldehyde(s) are produced following the oxidation of spermine by bovine serum amine oxidase. The involvement of these products in causing cytotoxicity was investigated in both drug-sensitive (LoVo WT) and drug-resistant (LoVo DX) colon adenocarcinoma cells, at 37 and 42°C. Cell survival experiments showed that LoVo DX cells are more sensitive than LoVo WT cells, at both temperatures. Cytotoxicity was considerably enhanced by hyperthermia. Treatments performed in the presence of catalase demonstrated that a marked reduction of the cytotoxic effect occurred in both cell lines at 37°C, apparently due to the clearance of H_2O_2 by catalase. Cytotoxicity was totally inhibited in the presence of both catalase and aldehyde dehydrogenase. Transmission electron microscopy studies showed ultrastructural alterations at mitochondrial level more pronounced in LoVo DX than in sensitive cells. Mitochondrial functionality studies performed by flow cytometry revealed a basal hyperpolarization of mitochondrial membrane in LoVo DX cells. The mitochondrial mass was evaluated after labelling the cells by NAO. The results showed that the higher cytotoxicity observed in LoVo DX cells was not due to a lower number of mitochondria, since in both cells lines comparable amounts of these organelles were present. After treatment with toxic products, an earlier and higher mitochondrial membrane depolarization was revealed in LoVo DX cells than in sensitive cells. These results support the hypothesis that mitochondrial functionality affects the cell sensitivity to the cytotoxic enzymatic oxidation products of spermine, which might be promising anticancer agents.

Rat cardiac polyamine metabolism response to HBO (hyperbaric oxygen) depends on intensity of treatment

S. Bettuzzi[1], **M. Friggeri**[1], **M. G. Troglio**[1], **F. Bacciottini**[1], **A. Caporali**[1], **S. Astancolle**[2], **D. D'Arca**[2], **A. E. Caccamo**[1], and **A. Casti**[1]

[1] Dipartimento di Medicina Sperimentale – Università di Parma, Via Volturno 39, Parma, Italy
[2] Dipartimento di Scienze Biomediche – Università di Modena e Reggio Emilia, Via G. Campi 287, Modena, Italy

Reactive Oxygen Intermediates (ROIs) are capable to interact with DNA, causing DNA fragmentation and apoptosis. Hyperbaric Oxygen (HBO) stress increases free oxygen availability, leading to increased production of ROIs. It is known that ROIs play a critical role in heart failure. In fact, myocardial cell survival depends on response to oxidative stress. Polyamines, and in particular spermine, could play a role in prevention of apoptosis and inhibition of DNA damage by acting as ROIs scavengers. Thus we studied cell proliferation and polyamine metabolism in the heart of rats subjected to short-time (15-min) or long-time (40-min) HBO treatment. In 15-min HBO treated rats, ODC mRNA and activity increased, paralleled by AdoMetDC activity, while SSAT mRNA decreased remarkably and OAZ mRNA did not changed significantly. This coordinated changes would likely cause an increased availability of spermine, the final product of polyamine biosynthesis that is suggested to exert a protective role against ROIs in myocardial cells. Histone H3 mRNA (a specific marker of the S-phase of the cell cycle) increased following a single 15-min treatment, suggesting that short-time exposure caused an early proliferative response in cardiac tissue. Longer exposure to HBO treatment (40-min) caused, instead, a decrease of the level of transcription of all the enzymes controlling the polyamine metabolism and H3, suggesting that only short-time exposure to HBO was capable to induce a compensatory mechanism, that was probably based on rapid synthesis of polyamines caused by coordinate induction of biosynthesis and inhibition of catabolism.

Effects of glucocorticoids on polyamine metabolism in some guinea pig tissues during sensitization

Gordana Bjelakovic, Dusica Pavlovic, Ivana Stojanovic, and **Jelenka Nikolic**

Institute of Biochemistry, Faculty of Medicine, University of Nis, Serbia

The usefulness of glucocorticoids in many immunological diseases has is well known. The idea of our work was to examine the metabolism of polyamines, spermine, spermidine and putrescine in liver and spleen tissues of sensitized guinea pigs under and without influence of dexamethasone, synthetic glucocorticoid. Sensitization of guinea pigs was done with application of Freund's adjuvant at the plantar parts of four legs. After that the animals are sacrificed at different period of time intervals (at sixth day, fourteenth, twenty first and twenty seventh day after antigen application). For biochemical investigation was used liver and spleen tissues. Examination of polyamines was done with butanol extraction followed by electrophoresis. Activities of polyamine oxidase and diamine oxidase have been measured by modified spectrophotometric method of Buchrach and Reches on the basis of determination of the formed amount of amino aldehydes. Proteins were measured according to Lowery method. Our obtained results indicate that the amounts of polyamines (spermine, spermidine and putrescine) augment during the time of sensitization in the liver and spleen tissues with the highest increase at twentieth day. Application of dexamethasone to sensibilized guinea pigs causes the opposite effect to spermine and spermidine – spermine amount increases but spermidine amount decreases. Polyamine oxidase activity follows the amount of spermine, the highest polyamine oxidase activity was noted with the highest amount of spermine.

Isolation and characterization of a rat hepatoma cell line resistant to the antiproliferative effects of agmatine

C. Cravanzola, G. Testore, S. P. Solinas, M. A. Grillo, and **S. Colombatto**

Università degli Studi di Torino, Dipartimento di Medicina e Oncologia Sperimentale, Sezione di Biochimica, via Michelangelo 27B, 10126 Torino, Italy

Agmatine, the product of arginine decarboxylation, was initially believed to be present in bacteria, plants and invertebrates only. Now it has been shown to be also present in mammals where it promotes a wide spectrum of physiopathological effects, including binding to imidazoline and α_2 receptors and inhibition of inducible NO synthase. Previously we have observed that agmatine transport into rat hepatocytes is mediated by the same carrier used by putrescine and that inside the cells it is in small amount hydrolysed to putrescine and urea and in a higher amount oxidised by diamine oxidase. In the meantime the intracellular content of polyamines is drastically decreased, due to overinduction of spermidine/spermine acetyltransferase and inhibition of ornithine decarboxylase. These modifications result in induction of apoptosis in not proliferating cells and in arrest of cell cycle in growing cells. By increasing agmatine concentrations stepwise (0.5 mM at a time) in the culture medium, a rat hepatoma cell line tolerant to chronic 2 mM agmatine concentrations was selected which showed viability and growth characteristics similar to those of the wild type hepatoma cells. Agmatine resistant cells cultured in the presence of different amounts of agmatine (0.5–1.0–2.0 mM) had a higher putrescine, spermidine and spermine content than parental cells cultured in the same conditions. Ornithine decarboxylase protein and activity were also increased, while spermidine/spermine acetyltransferase activity was reduced. Polyamines and agmatine were taken up in lower amount. Experiments by 2D electrophoresis of membrane proteins are under way to identify possible polyamine transporter(s).

Free-polyamine in tomato fruits after saline and nematocide treatments

P. Ruggeri[1], **C. Fabiano**[1], **E. Bellocco**[2], and **G. Laganà**[2]

[1] Departments of Environmental, Health, Social and Industrial Protection, Biochemical, Physiological and Nutritional Sciences, and
[2] Organic and Biological Chemistry, University of Messina, Italy

Polyamines are found in all organisms and are believed to be involved in several physiological processes in higher plants, including morphogenesis, rooting, flowering and senescence, in the plant response to abiotic stresses and in fruits development.

In this last case there are some evidences that fruits development and ripening are related to metabolic changes in polyamine levels.

Thus, several studies have indicated that changes in free polyamine levels may be an important part of the response mechanisms of plants to various stresses, such as K^+ deficiency, certain atmospheric pollutants and acidic, ionic, osmotic or water stresses.

Tomato development can be divided into 4 physiological steps: 1) fruit set induces cell-division-dependent growth lasting from 10 to 14 days; 2) cell expansion responsible for fruit size increase; 3) ripening starts after fruit reaches its final size, and 4) overripe stage when senescence starts.

In this study we tested the effect of saline stress and different nematocide (1,3-dichloropropene, methyl bromide) treatments on polyamine levels in tomato fruits (*Lycopersicon esculentum* cv. *Ikram*) grown in greenhouse.

Free-polyamines are extracted from mature fruits and determined by HPLC.

Results showed that saline stress and methyl bromide treatment induces a decrease of total free-polyamines vs. control fruits, whereas the treatment with 1,3-dichloropropene shows a slight dose-dependent increase in free-polyamine levels.

Involvement of polyamines in etoposide-induced apoptosis of mouse fibroblasts

E. Fiumana[1], **B. Tantini**[1], **M. Fattori**[1], **C. Pignatti**[1], **C. Stefanelli**[1], **C. Clô**[2], **F. Flamigni**[1], and **C. M. Caldarera**[1]

[1] Department of Biochemistry "G.Moruzzi", University of Bologna, Italy
[2] Department of Experimental Medicine, University of Parma, Italy

The polyamines putrescine, spermidine and spermine are absolutely required for cell growth and proliferation. However, polyamines have been recently implicated in the control of the apoptotic response. In fact excessive polyamine levels can directly trigger apoptosis, whereas polyamine depletion can either sensitise or protect cell exposed to death triggers.

Apoptosis is characterized by the activation of caspase proteases, that execute the death program.

In the present work we show that polyamines synthesis is necessary for caspase activation by the apoptosis-inducing drug etoposide in mouse fibroblasts. In order to study the mechanism(s) underlying the polyamine requirement for etoposide induced apoptosis, we also examined the role of polyamines in the signal transduction pathways activated by etoposide. The results indicate that etoposide elicited a sustained activation of extracellular signal-regulated kinase (ERK), a fundamental component of signalling pathways mainly required for cell proliferation, but in some system also for cell death. We found that ERK phosphorylation is strongly correlated with caspase activation and that both events are prevented in polyamine depleted cells. We also show that NF-κB, a key transcription factor involved in cell proliferation as well as in the control of apoptotic pathways, may play a role in the impaired response to etoposide provoked by polyamine depletion.

In conclusion our results suggest that polyamines play a permissive role in the pathways triggered by etoposide and leading to cell death of fibroblasts, by supporting the activation of ERK, NFκB and caspases.

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Interference with histidine decarboxylase activity during pregnancy associated growth of mammary gland

Waldemar Wagner and **W. Agnieszka Fogel**

Polish Academy of Science, Institute of Biogenic Amines, Lodz, Poland

Both histamine and polyamine systems are involved in tissue growth and differentiation. In this abstract, we present data suggesting interactions of these systems in developing mammary gland exposed to reduced histamine synthesis. This was achieved by targeting histidine decarboxylase (HDC) mRNA with phosphorothioate antisense oligodeoxyribonucleotides. The antisense oligonucleotides covered 62–90 (5′–3′) fragment of HDC mRNA and their efficiency was successfully verified in mammary epithelial cell cultures prior to *in vivo* experiments. Mice from $10–12^{th}$ day until $17–19^{th}$ day of pregnancy were given continuous infusions of either antisense or corresponding sense oligonucleotides, via subcutaneously implanted miniosmotic pumps (Alzet 1007D) at a delivery rate of 5.25 mg/kg/24 h. As controls corresponding pregnant and adult virgin mice were employed.

Antisense oligonucleotides treated pregnant mice expressed roughly 60% lower mammary HDC activity; their tissue HDC transcript was reduced to 67% whereas ornithine decarboxylase mRNA was 86% of the control. Neither HDC activity nor HDC mRNA was altered by sense oligonucleotides treatment. However, ornithine decarboxylase mRNA was increased by approximately 50%. Both histamine H_1 and H_2 receptor mRNAs were examined and the former was undetectable whereas the latter up regulated in the mammae of pregnant antisense oligonucleotide treated mice. Morphological examination revealed no gross changes after treatment however adipocytes were more pronounced and the number of lymphocytes infiltrating the mammary tissue increased.

The data indicate cross talk of histamine and polyamine systems and are compatible with proliferative effects of histamine via H_2 receptor.

Cellular localization of polyamine transporters TPO1 to TPO4 and regulation of TPO1 activity by phosphorylation

K. Kashiwagi, T. Uemura, H. Tomitori, K. Tachihara, and **K. Igarashi**

Graduate School of Pharmaceutical Sciences, Chiba University, Chiba, Japan

Cellular localization of TPO1, TPO2, TPO3 and TPO4 proteins of *Saccharomyces cerevisiae* was determined by sucrose density gradient fractionation of membranes and indirect immunofluorescence microscopy of cells. When TPO1 to TPO4 were expressed with a multicopy vector, these proteins were mainly located on the plasma membrane, but some of them were located on the vacuolar membrane. This was also suggested by the results that spermine uptake by TPO1 to TPO4 was significantly inhibited by bafilomycin A_1, a specific inhibitor of vacuolar H^+-ATPase. It was found that both resistance to spermine toxicity and increase in spermine uptake activity by TPO1 was greatly diminished by the mutation of Thr^{85} to alanine. Thr^{85} is a putative phosphorylation site by cAMP-dependent protein kinases. Resistance to spermine toxicity was recovered by the mutation of Thr^{85} to glutamate or aspartate. The results suggest that TPO1 activity may be regulated by cAMP-dependent protein kinases by phosphorylating the Thr^{85} residue. When spermine toxicity was compared in three yeast strains deficient in cAMP-dependent protein kinases (1 to 3), two of them (1 and 2) became sensitive to

spermine toxicity. The results suggest that cAMP-dependent protein kinases 1 and 2 are involved in the activation of TPO1 activity.

Biogenic amines and apoptosis

A. Toninello[1], **M. Salvi**[1], **P. Pietrangeli**[2], and **B. Mondovì**[2]

[1] Department of Biological Chemistry and CNR Center for the Study of Biomembranes, University of Padua, Padua, Italy
[2] Department of Biochemical Sciences "A. Rossi Fanelli" and C.N.R. Institute of Molecular Biology and Pathology, University of Rome "La Sapienza", Rome, Italy

It is well known in the literature that polyamines are involved in cell growth and, consistent with this role, their ability to protect cells from apoptosis. However, polyamines, also have a role in facilitating cell death. In this context, the oxidation of spermidine and spermine either by FAD or copper-dependent amine oxidases produces H_2O_2 (and their reactive oxygen species, ROS) and aldheyde which are strong inducer of apoptosis.

Taking together, these facts indicate a role of polyamines and other biological active amines in protection or damage of cells.

H_2O_2 and aldehydes are involved in the induction and/or amplification of the mitochondrial permeability transition (MPT). This phenomenon, which provokes a bioenergetic collapse and a redox catastrophe, is strongly inhibited by polyamines in isolated mitochondria. Monoamines instead exhibit an inhibitory effect at higher concentrations, while at low concentration behave as inducer agents. MPT is characterized by the opening of a channel, the transition pore, which permits a non specific bi-directional traffic of solute across the inner membrane, leading to swelling of the organelle and release of cytochrome c and apoptosis-inducing factor. These proteins, in turn, activate the caspase-cascade, which triggers the apoptotic pathway. Depending on their cytosolic concentration, metabolic conditions and cell type, polyamines act as promoting, modulating or protective agents in mitochondrial-mediated apoptosis. While their protective effect could reflect inhibition on MPT induction and retention of cytochrome c, the promoting effect can be explained by the generation of ROS that induce the apoptotic effect mediated by MPT and cytochrome c release.

Polyamine biosynthetic pathway of an extreme thermophile, *Thermus thermophilus*

Mio Ohnuma[1], **Yusuke Terui**[1], **Mizue Nagai**[1], **Hiromi Ito**[1], **Masatada Tamakoshi**[1], **Masaru Niitsu**[2], **Keijiro Samejima**[2], and **Tairo Oshima**[1]

[1] Department of Molecular Biology, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo, 192-0392, Japan
[2] Faculty of Pharmaceutical Sciences, Josai University, 1-1 Keyakidai, Sakado, Saitama 530-0248, Japan

Thermus thermophilus produces 16 kinds of polyamines including putrescine, spermidine, and spermine. The other uncommon 13 polyamines include unusually long polyamines such as pentamines and hexamines, and branched ones in addition to relatively distributed polyamines such as thermine and homospermine. These long and branched polyamines have a marked effect on protecting DNA and activating cell-free polypeptide synthesis. The polyamine composition of *T. thermophilus* cells varies depending on growth temperature: cellular contents of longer and branched polyamines increase at higher temperature. Therefore, long and branched polyamines may play important roles in various cellular functions at high temperature. To elucidate the biosynthetic pathway of uncommon polyamines of *T. thermophilus* and the effects on cellular functions, we cloned polyamine biosynthetic genes for biochemical and genetic analyses.

Homologs of known polyamine biosynthetic enzymes except for ornithine decarboxylase were found in *T. thermophilus* genome by homology search. We disrupted some of these genes of *T. thermophilus* and analyzed intracellular contents of polyamine. The disruption of agmatine ureohydrolase homolog resulted drastic reduction of triamine, tetraamine and longer polyamines. In addition, agmatine was not accumulated in the cells. The disruption of putrescine aminopropyltransferase homolog resulted in increase of diamine and triamine and disappearance of tetraamine and longer polyamines. Biochemical analyses of the putrescine aminopropyltransferase homolog revealed that this enzyme utilizes triamine as substrate instead of diamine. These results indicate that in *T. thermophilus*, putrescine is mainly derived from arginine, and longer and branched polyamines are products of the putrescine aminopropyltransferase homolog.

Reverse genetic studies on roles of unusual polyamines in *Thermus thermophilus*

M. Ohnuma[1], **H. Ito**[1], **Y. Terui**[1], **M. Tamakoshi**[1], **M. Niitsu**[2], **K. Samejima**[2], and **T. Oshima**[1]

[1] Department of Molecular Biology, Tokyo University of Pharmacy and Life Science, Hachioji, Tokyo 192-0392, Japan
[2] Faculty of Pharmaceutical Sciences, Josai University, Sakado, Saitama 530-0248, Japan

An extreme thermophile *Thermus thermophilus* produces many unusual polyamines including long polyamines such as caldopentamine or caldohexamine and branched polyamines such as tetrakis(3-aminopropyl)ammonium. To understand the role of these unusual polyamines in physiology at elevated temperature, some genes involved in polyamine biosynthesis were knocked out, and the resulted mutants were analyzed. Homology search on the whole genome sequence of the extreme thermophile revealed the lack of *speC*, the gene for the key enzyme (ornithine decarboxylase) of polyamine metabolisms in many organisms. Homologues of *speA*, *B*, *D*, *E* were found in the genome sequence suggesting that putrescine is produced via agmatine in the thermophile.

We knocked out *speA* and *speE* genes respectively. Mutant thermophiles, TtspeA and TtspeE respectively, were able to grow in a synthetic medium at 75°C. Both mutants did not produce longer polyamines and tetrakis(3-aminopropyl)ammonium. Instead, a large amount of a triamine was present in the cells; the triamine was tentatively speculated to be homospermidine in case of TtspeE, and aminopropylagmatine in case of TtspeA. Currently we are trying to identify the chemical structures of these triamines. Both mutants lost two microbiological features; (1) growth at 80°C is poorer than that of the wild type strain, and (2) production of carotenoid dyes. These results suggest that (1) at relatively lower temperatures, triamines can fill in for the physiological roles of longer or branched polyamines, (2) longer polyamines and/or the branched quaternary amine is essential for both the growth at higher temperatures and biosynthesis of carotenoids in the thermophile.

Protective effect of spermine on hepatic injury in the iron-overloaded rats

D. Pavlovic, G. Kocic, G. Bjelakovic, I. Stojanovic, D. Sokolovic, and **B. Djindjic**

Institute of Biochemistry, Faculty of Medicine, Nis, Serbia and Monte Negro

Oxidation of polyamines by polyamine oxidase (PAO) is a well-known cause of polyamine cytotoxicity but spermine oxidation-mediated apoptotic cell death mechanism involved remains unclear. PAO catalyzes oxidative deamination of polyamines, the cellular aliphatic cations. This reaction produces highly toxic hydrogen peroxide, 3-acetamidopropanal, and precursors of higher polyamines.

Aim: Based on the fact that spermine might have a modulating role on oxidative stress as well as that it may form an unreactive chelate with Fe a possible link between iron overload liver oxidative damage and spermine oxidation is discussed.

Methods: The Wistar rats were allocated to the following groups: I-controls; II-treated with a single dose of $FeSO_4$ (3 mg/kg BM, ip); III-treated with $FeSO_4$ and spermine (500μmol/kg BM, ip) simultaneously; IV-treated with spermine only. Rats were killed 3 h after. The content of malondialdehyde (MDA), protein carbonyl groups and iron content were measured as parameters of oxidative liver injury.

Results: In $FeSO_4$-treated rats a marked increase in the hepatic level of iron (0.89 ± 0.23 g/mg prot vs. control 0.49 ± 0.17 $p < 0.001$) was associated with enhanced MDA level (2.13 ± 0.5 vs. control 1.3 ± 0.3 nmol/mg prot $p < 0.01$) and increased PAO activity (2.75 ± 0.3 vs. control 1.83 ± 015 $p < 0.001$) respectively. In group treated with $FeSO_4$ and spermine, iron content (0.36 ± 0.07 $p < 0.01$) as well as PAO activity (2.01 ± 0.2, $p < 0.001$) were significantly decreased compared with Fe treated only and carbonyl group content tended to be lower in comparison to $FeSO_4$ treated only (1.58 ± 0.24), but MDA level did not change (2.31 ± 0.72) In addition, treatment with spermine alone resulted in an increase of MDA level (2.74 ± 0.7 vs. control $p < 0.01$), as well as PAO activity (2.26 ± 0.3 vs. control $p < 0.01$), iron content did not change (0.59 ± 0.29), but carbonyl groups were decreased (0.99 ± 0.28 vs. control $p < 0.05$).

Conclusions: Fact that the polyamine depletion induced the stress-activated protein kinase (JNK) type of mitogen-activated protein kinase (MAPK) rasing the possibility that polyamine catabolism observed in iron overload may directly suppress liver regeneration and repair as well as alter cellular signaling pathways including caspase activating apoptosis signal cascade. Obtained results suggest that modulation of iron overload-mediated liver injury by spermine might represent a promising area for further research and therapeutic manipulation.

Arginine and polyamine metabolism in mouse kidney: influence of gender and dietary arginine

Rafael Peñafiel, Carolina Ruzafa, Francisco Monserrat, and **Asunción Cremades**

Departamentos de Bioquímica y Biología Molecular y Farmacología, Facultad de Medicina, Universidad de Murcia. Campus de Espinardo, 30100 Murcia, Spain

The kidney plays a central role in arginine metabolism, not only because most known enzymatic reactions involving L-arginine as substrate take place in renal tissue but because this organ is essential for arginine synthesis in many animal species. We report the existence of a marked sexual dimorphism in arginine content in plasma, kidney and skeletal muscle of CD1 mice which is influenced by arginine dietary content and that is also associated to gender-dependent differences found in the activities of several renal enzymes implicated in arginine and polyamine metabolism. In fact, dietary arginine restriction produced a higher decrease in arginine concentrations in females than in males, a reduction in arginase and nitric oxide synthase in the females and in ornithine decarboxylase and putrescine excretion in the males. We also tested the renal expression of arginine descarboxylase, the agmatine-forming enzyme recently associated with polyamine metabolism in mammals. Although a gender-dependent arginine decarboxylation was observed in different renal fractions *in vitro*, agmatine formation could not be detected either by HPLC or by paper electrophoresis. Our results indicate that dietary arginine plays a relevant role in the maintenance of arginine sexual dimorphism, and that the pathway leading to the conversion of arginine into agmatine appears to be non-operative in the mouse kidney. These findings may have physiological significance because of the important effects that arginine-derived products exert on a variety of cellular processes.

Polyamine levels in a group of free-living amoebae, negleria and acanthamoeba spp.

P. Ruggeri[1], **D. Forino**[1], **R. Leonaldi**[1], **C. Fabiano**[2], **D. Caccamo**[2], and **R. Ientile**[2]

[1] Departments of Environmental, Health, Social and Industrial Protection, Social Medicine, and

[2] Biochemical, Physiological and Nutritional Sciences, University of Messina, Italy

The ubiquitous occurrence of the aliphatic polyamines putrescine, spermidine and spermine, and their own role in cell proliferation and differentiation are just documented. It was shown that polyamines also play a role in the growth of protozoal parasites, e.g., trypanosomes and various species of Leishmania, in which they show their maximal levels during the logarithmic phase of growth.

Negleria and Acanthamoeba species, a widely distributed group of free-living amoebae, can infect humans and spread hematogenously after direct interaction with the mucosal surface, and may cause chronic central nervous system infections or eye infections.

Granulomatous amebic encephalitis due to Acantamoeba spp. usually occurs in chronically ill and debilitated peoples, or in patients with alteration of the immune system, like immunodeficiency syndrome (AIDS). In contrast, primary amebic meningoencephalitis due to Naegleria spp. usually occurs in healthy men, young peoples with a swimming history in heated swimming pools or in manmade lakes, or peoples who play water-related sports.

In this paper we describe the polyamines pattern in free-living ameba species, grown in presence or absence of polyamines.

Polyamines were extracted from pellets of trophozoites of Acanthamoeba castellani, Acanthamoeba russ, Acanthamoeba polyphaga, Naegleria australiensis, Naegleria lovaniensis, by 3% PCA and their benzoil derivatives were separated, identified and quantified by HPLC. Results obtained demonstrate a difference in the polyamine pattern of the amoeba strains examined and in some of them 1,3-diaminopropane as the major product.

When we add polyamines in medium, the previous described differences increase.

Evidence of conformational changes in mammalian histidine decarboxylase during catalysis: a new target for selective inhibition

Carlos Rodríguez Caso[1], **Daniel Rodríguez-Agudo**[1], **Aurelio A. Moya-García**[1], **Miguel Ángel Medina**[1], **Vinod Subramaniam**[2], and **Francisca Sánchez-Jiménez**[1]

[1] Department of Molecular Biology and Biochemistry, Faculty of Sciences, Campus de Teatinos, 71 Málaga, Spain

[2] Department of Molecular Biology. Max Planck Institute for Biophysical Chemistry, Am Fassberg 11, D-37077 Goettingen, Germany

Mammalian histidine decarboxylase is synthesized as a 74 kDa precursor, which has to be processed to yield the 53–58 kDa monomers of the mature, active dimeric enzyme. As a model to study structure/function relationships of the mature histidine decarboxylase, we are using a recombinant version of a carboxy-truncated form of the rat enzyme (1/512 fragment), which has kinetic constants similar to the mature enzyme purified from rodent tissues. We hypothesized that the known conformational change of the coenzyme during catalysis could be accompanied by a conformational change of the polypeptide structure. To test this hypothesis, we have carried out spectroscopic and electrophoretic analyses of purified recombinant rat 1/512 histidine decarboxylase in the presence and absence of substrate or substrate analogues able to block the catalytic site at different catalytic steps, allowing to analyse the respective conformational states of the protein along reaction. Results

are discussed on the bases of a biocomputational 3D model of the dimeric enzyme. Stability of these conformational states against different structural disruptor agents has also been studied. All results together indeed indicate that the enzyme adopts different conformations during reaction, providing insights for development of new antihistaminic strategies.

The role of spermidine in the greening process stimulated by cytokinin

E. A. Sobieszczuk[1], **A. Di Sandro**[2], and **J. Legocka**[1]

[1] Faculty of Biology, Laboratory of Plant Physiology, Adam Mickiewicz University, Al. Niepodległości 14, 61-713 Poznań, Poland
[2] Dipartimento di Biologia e.s, Universita degli Studi di Bologna, Via Irnerio 42, 40126 Bologna, Italy

The present work was performed in order to evaluate the possible relationship between cytokinin and polyamines (PAs) during one of the well-documented responses of cytokinin stimulation of chloroplast ontogenesis.

We examine the effect of kinetin on the changes in the level of free PAs in cotyledons and polyamines bound to thylakoids membranes and to PSIIα in excised cucumber cotyledons during greening process. Kinetic analysis of polyamines content revealed that the level of free Spd in cotyledons incubated on light increased till 4 h and then decreased. Kinetin enhanced this process. To verify if the decrease observed was due to oxidation or conjugation, PAO and thylakoids TGase activity were measured. Only the latter increased.

Spd was the dominant polyamine bound to thylakoids membranes. After 6 h incubation on light the level of bound Spd was almost two times higher for cotyledons incubated with kinetin in respect to the control. We also observed that Spd was conjugated via TGase to thylakoid membranes and Spd was favoured in the conjugation in comparison with Put.

The effect of kinetin on the level of SAMDC mRNA transcript and enzyme activity was studied. Our results suggest that light induces the gene expression for SAMDC and cytokinin elevates and accelerates this process about 2 h.

Our results show that cytokinin-induced chloroplast differentiation in cucumber cotyledons may be partly mediated by the enhanced synthesis of PAs and by stimulation of PAs binding to thylakoid membranes, thus stabilising their structure.

Cystamine induces apoptotic death in rat hepatoma cells

E. Amodeo, S. Colombatto, and **S. P. Solinas**

Dipartimento di Medicina e Oncologia Sperimentale dell'Università degli Studi di Torino, Sezione di Biochimica, Italy

Cells have efficient defensive mechanisms to prevent injury induced by several reactive oxygen species produced during normal metabolic pathways. Oxidative damage occurs when oxygen reactive species overwhelm the cellular biochemical defences. Therefore while some cells die by necrosis, on the contrary others die by apoptosis. The last one is particularly interesting because it includes a series of distinctive morphological and biochemical alterations. It has been widely accepted that cystamine, a product of the pantothenate metabolism, is oxidatively deaminated to cystaldimine producing hydrogen peroxide, being the last one the most significant oxidative compound. Consequently we have supposed that cystamine metabolism might give rise to oxidative stress and, in alternative or in addition, cystamine itself might be an inhibitor of some enzyme involved in cellular defence.

As preliminary results showed that cystamine induces apoptosis in rat hepatoma cells, we are investigating on the mechanism of specific cystamine-induced apoptotic death in this cell line. Our data demonstrate that cystamine treatment, despite being a caspase inhibitor (as confirmed in literature), induces nuclear fragmentation and externalization of membrane phosphatidylserine. These are general features of apoptosis. We have also observed that the pentose phosphate pathway and the release of anione superoxide, induced by menadione, increase in cystamine treated cells respect to the controls. Moreover cystamine treatment increases total glutathione content, while superoxide dismutase and glutathione-S-transferase activities are not significantly changed.

All these effects emphasize an interesting behaviour of cystamine as inductor of apoptosis.

Polyamine and nitric oxide metabolism interplay changes during experimental epilepsy in different brain regions

I. Stojanovic, A. Jelenkovic, I. Maksimovic, G. Bjelakovic, and **D. Pavlovic**

Institute of Biochemistry, Medical Faculty, University of Nis, Serbia and Montenegro

Human status epilepticus is consistently associated with cognitive problems and widespread neuronal necrosis. The molecular mechanisms underlying neurophysiological and neurochemical changes, as well as increased excitability in human epilepsy, are still unknown. In animal models convulsive status epilepticus also leads to extensive neuronal necrosis in vulnerable brain regions.

Electroconvulsive shock or agents producing seizures caused marked changes in brain polyamine metabolism. Polyamines are thought to be involved in modulation of NMDA receptor neurotransmission during convulsions of different origin. Alterations of polyamine metabolism during seizures are differently pronounced in different brain regions and the literature data are contradictory.

Nitric oxide (NO) is intercellular messenger which plays the role of universal modulator of various physiological functions in nervous system such as interneuronal communications, synaptic plasticity, memory formation, receptor functions, intracellular transmission, release of neurotransmitters. It is produced in neurons, glia cells and vascular endothelium expressing, depending on origin, neuromodulatory or neurotoxic effects. It is believed to be a key pathophysiological factor of CNS diseases associated with the neurotoxic effects of glutamate (ischemia, stroke, convulsive disorders). In convulsions it mediates the increase in local cerebral blood flow and is reported to have either pro-or antiepileptic effects.

Since polyamines and nitric oxide share the same substrate, arginine, and also, can influence each other's metabolism depending on the conditions, the aim of this study was to examine the interdependence of their metabolism and effects during experimentally induced seizures in the conditions of different availability of arginine and upon influence of putrescine pretreatment.

Regulation of cell proliferation and tumor growth processes by chemical compounds mediated by polyamines metabolism

S. P. Syatkin[1], **T. V. Fedorontchouk**[1], **K. E. Golamazova**[1], **E. A. Miroshnicova**[1], **N. Ja. Greedina**[2], **M. Yu. Lidack**[3], and **T. T. Berezov**[1]

[1] Russian People's Friendship University, Moscow, Russia
[2] R.E. Kavetsky Institute of Oncology Problems, Ukrainian Academy of Sciences, Kiev, Ukraine
[3] Institute of Organic Synthesis, Riga, Latvia

The metabolism of PA were studied in transplanted hepatomas 27, 22a, 60, 61, 46, and 48 and in the course of hepatocarcinogenesis

induced by nitrosodiethylamine in comparison with normal and regenerating livers. The data obtained suggest that there are different mechanisms of elevation of PA levels in indicating tissues. Thus the accumulation of PA in tumor tissues are probable due to the sharp decrease or loss of diamine oxidase (DAO) activity rather than that by alterations of ornithine decarboxylase (ODC) activity. In regenerating rat liver it depends from the increasing activity of ODC, while the DAO activity remained constant. 26 novel chemically modified PA analogs were evaluated for their ability to inhibit the PA biosynthesis and to promote the PA degradation in two cell-free systems as well as on the growth of L- and CaOv tumor cells. Bis(uracilyl)-analogs and 8-(2-oxyethyl)amino-9-β-D-xylofuranosyl-adenine suppressed PA and putrescine synthesis and in the same conditions were more effective than DL-α-difluoromethylornithine (DFMO) – strong specific inhibitor of ODC. The other adenosine modified compounds could act both as activators of ODC and inhibitors both diamine and polyamine oxidase activities in regenerating liver test system. In contrast to those mentioned above two uracils modified agents as well as DFMO were able to inhibit ODC and to increase the rate of oxidative deamination of PA in the same system. Thus bis(uracilyl) – PA analogs were the most active and it was proposed to further investigation as substances having potential antitumor and antiproliferative properties.

Proteomics

Functional proteomics of signal transduction pathways

J. Godovac-Zimmermann

Department of Medicine, University College London, 5 University Street, London WC1E 6JJ, UK, ProteoSys AG, Mainz, Germany

One of the most intellectually challenging, scientifically productive, and socially applicable endeavours of modern biological science is the current effort to understand the function of living cells at the molecular level.

We are currently witnessing an emergence of a new view of cellular biology that emphasized the need for quantitative measurements of networks and fluxes. As very large portions of cellular networks and fluxes will not be accessible to analysis with genomics methods, the tools such as functional proteomics will be an integral and essential part of this endeavour.

Several kinds of experiments have been carried out successfully in our labs. (1) Identification of post-translational modifications of the endothelin A and B receptors (ETAR and ETBR) including both phosphorylation and acylation. We have developed new, very efficient methods for single step isolation of highly pure ETAR and ETBR from cells. This has allowed us to obtain evidence that the post-translational modifications are very complex and result in multiple phenotypes showing different forms of modification for receptor. As with other systems, e.g. insulin-like growth factors, it is probable that these multiple phenotypes of the ET receptors correspond to different forms of signalling dependent on cellular state, e.g. the cell cycle. It is, for example, already clear from the phosphorylation of the receptor that a series of different kinases must be involved. (2) Following stimulation of fibroblasts with endothelin, phosphorylation/dephosphorylation signalling cascades involving several hundred proteins have been observed by use of high resolution 2D electrophoresis and detection of phosphorylated proteins labelled with ^{32}P by autoradiography or immunological methods. The large number of proteins involved are being identified by mass spectrometric methods such as mass fingerprinting or sequencing by mass spectrometry. (3) Differential gene expression has been followed by using ^{35}S Met pulse chase labelling concurrently with endothelin stimulation. At least 50 proteins showed significant changes in expression on 2D gels and these proteins are also being identified. (4) We have developed a platform for automated high-throughput analysis of phosphoproteins for proteomics analysis of complex cellular signalling networks involving multiple, time dependent protein phosphorylation events. (5) Ultra-high sensitivity ^{125}-Iodine and ^{13}1-Iodine labelling of proteins together with multi-photon-detection (MPD) imaging has been used to detect changes in protein levels in cancer tissues. 10 attomoles of proteins could be routinely and quantitatively detected on 2D electrophoresis gels with a linear dynamic range over six orders of magnitude of protein concentrations. Protein levels were measured with as little as one protein copy per cell.

These experiments demonstrate that it is now possible to use proteomics methods to investigate the integration of response to an extracellular signal at the levels of the receptor itself, the subsequent signalling cascades and the ensuing gene expression. The proteomics technology permits concurrent monitoring of large numbers of protein phenotypes (the forms and amounts of individual proteins and is therefore able to provide a global overview of signalling processes which greatly augments more traditional investigations of individual proteins or pathways. Furthermore, these new methods will allow quantitative determination of the changes in protein phenotypes, which is very important in view of the highly non-linear amplification properties of such signalling processes.

Proteomics in cancer research

Luca Bini, Sabrina Liberatori, Laura Bianchi, Eloise Mastrangelo, Chiara Tani, and **Vitaliano Pallini**

Functional Proteomic group, Department of Molecular Biology, University of Siena, Siena, Italy

Proteome analysis is aimed mainly to identify and characterize proteins expressed in tissues and body fluids. Theory and techniques for the description of proteome (PROTEins expressed by a genOME) have been developed during the last decade with the aim to define as many as protein components of cells and tissues, deriving from gene expression and subsequent modifications at the post-transcriptional (alternative splicing) and post-translational levels (e.g. phosphorylation, glycosylation, etc.).

Cancer in humans results from complex gene interactions, different cellular events and environmental influences. Therefore, a precise diagnosis and prognosis in a proliferative disorder will depend on the study of epigenetic regulation of tissue survival and growth for each patient. For this reason a proteomic approach could be very helpful to have a complete view of the protein changes involved in cancerous process. We will present results obtained in our laboratory, studding breast cancer in order to identify cancer-associated proteins to be used in diagnosis and/or prognosis.

Proteomic profiling of Pseudoxanthoma elasticum fibroblasts

P. Cancemi[1], **S. Caricato**[2], **S. Minafra**[2,3], and **I. Pucci-Minafra**[2,3]

[1] Department of Cell Biology and Development,
[2] Center of Experimental Oncobiology, and
[3] Department of Experimental Oncology and Clinical Applications, University of Palermo, viale delle Scienze, 90128, Palermo, Italy

Pseudoxanthoma elasticum (PXE) is an inherited disorder of the connective tissue, characterised by progressive mineralization of elastic fibers, alteration of collagen fibrillogenesis and accumulation of molecules in the extracellular space. Clinical manifestations involve skin,

eyes, the gastrointestinal and the cardiovascular system. The severity of the disease may vary within the same family. Recently, PXE has been linked to mutations in the MRP6/ABCC6 gene, a member of the ABC transporter family, expressed primarily in the liver and the kidneys. The normal function of MRP6, including its physiological substrate(s), is still unknown. In order to better understand the biological roles and to identify the targets of these genes the proteomic approach seems to offer great opportunities. Thus, the aim of the present study was to compare the proteomic and enzymatic profile of *in vitro* cultured fibroblasts from PXE patients and from healthy subjects. Protein present in the cell layer, as well as those released in the culture medium, were separated by 2D-IPG and then identified by gel matching, immunodetection, Nt-micro-sequencing, and by zymography for the culture media. The relative levels of spots intensity were determined by computer-assisted analysis.

Results indicate that major differences in the protein expression profile between normal and PXE fibroblasts were related to some metabolic enzymes, cytoskeletal proteins, including different forms of vimentin, and in phosphorylation status of a number of proteins. We also observed different levels of matrix protease activities in culture media from normal and PXE subjects.

De novo proteins on the base of albebetin and functional fragments from α_2-interferon, differentiation factor HLDF and insulin: design and properties

R. V. Chertkova[1], **V. P. Zav'yalov**[2], **I. A. Kostanyan**[1], and **D. A. Dolgikh**[1]

[1] Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, ul. Miklukho-Maklaya 16/10, Moscow, 117997, Russia
[2] Branch of biomedicine technologies, Russian University of Friendship, ul. Miklukho-Maklaya 6, Moscow, 117198, Russia

Functional peptide fragments from human α_2-IFN, differentiation factor HLDF and insulin corresponding to the functionally important sites of their molecules were grafted into *de novo* protein albebetin engineered to attain a pre-designed tertiary structure with a unique topology that has not been observed in natural proteins. By means of genetic engineering the DNA fragments corresponding to these peptides were inserted into the albebetin gene to obtain two variants of albebetin with fragments of human α_2-IFN (LKDRHDF (30–36) and LKEKKYSP (130–137), albebetin with peptide fragment TGENHR (41–46) from differentiation factor HLDF and two variants of albebetin with insulin-like peptid GERGFFYCN. The chimerical genes were expressed in *Escherichia coli* in a fusion expression system with thioredoxin. The fusion proteins were digested by highly specific protease "factor Xa" and the target chimerical proteins were purified by ion-exchange and metal-affinity chromatography and tested for their structure and biological activity.

According to the CD and ^{1}H-NMR spectroscopy study the chimerical proteins maintained the pre-designed structural properties of albebetin. Antiviral activity of *de novo* proteins with human α_2-IFN fragments was studied *in vitro* using cell lines L-41 (human mononuclear leucosis) and VERO (green simian kidney). Treatment of these cell lines with the proteins revealed the direct dose-dependent antiviral activity on both cell cultures, one of the proteins being almost as active as α_2-IFN. The proteins reveal cytotoxicity only at concentrations exceeding minimal active concentration by 6 orders of magnitude. Albebetin with peptide fragment of differentiation factor HLDF acquired ability to stop proliferation and to differentiate the HL-60 leukemia cells almost as efficiently as the differentiation factor itself. One of two *de novo* proteins including insulin-like fragment acquired ability to stimulate glucose uptake by L-929 cells although the efficacy of stimulation was lower than that for the synthetic peptide and insulin.

These results demonstrated that albebetin could be used as a scaffold for constructing of the functionally active *de novo* proteins possessing the pre-designed tertiary fold of albebetin and various biological activities.

Plant pathogens and defence responses to infection: crystallographic studies

Luca Federici[1], **Adele Di Matteo**[2], **Benedetta Mattei**[3], **Claudio Caprari**[3], **Felice Cervone**[3], and **Demetrius Tsernoglou**[2]

[1] Department of Biochemistry, University of Cambridge, UK
[2] Department of Biochemistry and
[3] Department of Plant Biology, University of Rome "La Sapienza", Italy

The cell wall is the first barrier that plant cells use to oppose to the attack of pathogens. Most phytopathogenic microorganisms produce enzymes that degrade cell wall polymers. Among these, polygalacturonases are secreted by phytopathogenic fungi at the very early stages of plant infection. They hydrolyse the pectin component of the plant cell wall, favouring fungal hyphae penetration and tissue colonization. To counteract the enzymatic hydrolysis of pectin, most plants express extracellular proteins that specifically bind and inhibit fungal polygalacturonases. Polygalacturonase-inhibiting proteins (PGIPs) belong to the leucine-rich repeat family of proteins and are homologous to the plant resistance genes products. The interaction limits the aggressive potential of polygalacturonases and results in the accumulation of molecules which act as elicitors of plant defence responses. Interestingly a high degree of polymorphism is found both in polygalacturonases and PGIPs, accounting for the specificity of different inhibitor isoforms for polygalacturonases from different fungi. We have determined the crystal structures of the polygalacturonase of the phytopathogenic fungus *Fusarium moniliforme* and of a bean PGIP. Moreover we mutated several residues of both proteins and measured the affinities of the native and variant complexes. These data enable us to discuss the mechanism for the complex formation and enzyme inhibition and to start addressing the question of different specificities. A comprehension of the molecular basis of polygalacturonase-PGIP interaction is crucial for dissecting the role of PGIPs in plant defence and for aiding the design of inhibitors with a broader spectrum of interaction, useful for crop protection.

Proteomic modulation in breast cancer cells

Simona Fontana[1], **Patrizia Cancemi**[2], **Salvatore Minafra**[1,3], and **Ida Pucci-Minafra**[1,3]

[1] Department of Experimental Biology and Clinical Applications,
[2] Department of Cell Biology and Development, and
[3] Center of Experimental Oncobiology; University of Palermo, viale delle Scienze, 90128, Palermo, Italy

During cancer progression, neoplastic cells reach underlying stroma, establish new and dynamic contacts with extracellular matrix (ECM) and are exposed to signals that influence their proliferation, adhesion and migration. Moreover during invasive growth of malignant carcinomas, underlying ECM undergoes some local and progressive modifications. We had previously demonstrated that in both breast and colon carcinomas stroma, a new form of embryo-foetal collagen (OF/LB) and an increased amount of type V collagen are deposited. "*In vitro*" studies showed that OF/LB collagen exerts a permissive effect on proliferation and migration of breast cancer cells (8701-BC), while type V collagen has an inhibitory effect on these cells. The aim of the present research was to investigate further, at the proteomic level, the effect of OF/LB and type V collagens used as substrates for neoplastic cell growth. Due to the complexity of a whole proteomic profile, subsets of 58 proteins were

used as markers to assess possible variations in the protein expression. For this study we adopted a multivariate statistical procedure that allows to maintain a global view of the variations induced by different growth condition, when several variables have to be analysed simultaneously.

The results of this research indicate that in function of growth substrates, chaperons and heat shock proteins most contributed to the dissimilarity in levels of expression of selected spots; moreover we observed that different isoforms of the same protein showed independent levels of expression from one another in function of the growth substrates.

Phage-display mimotope-peptide approach in structural study of helical proteins

K. G. Gazarian, T. G. Gazarian, C. F. Solís, and **J. P. Laclette**

Instituto de Investigaciones Biomédicas, UNAM, México D.F. México

Peptide phage display is powerful methodology for epitope discovery. By localizing them, the method yields important information on the fine primary, secondary, tertiary and quaternary protein structure. This potential of the methodology is not yet widely used in proteomic studies because the approach is in its technical ''infancy'', requiring special studies on protein models of each type structure for better understanding the relationships between mimotopes, epitopes and protein structure. Secondary structure is the best among protein elements to start such analysis since, first, conformationally is made of a few configurations (helices, β-structures, turns), and, second, fibrous protein monomers adopt exclusively secondary structure, whose specific relationships with epitopes can be revealed. Using, 862aa paramyosin of parasite cestode *Taenia solium* as a model of a-helical proteins, we developed a strategy of phage display analysis of relationships between the critical amino acids of the mimotope-identified epitopes and the protein residues with the propensity of forming helical versus non-helical, conformation. The strategy includes: (a) the use of synthetic peptides of computer algorithm-predicted protein immunogenic sites for generating antibodies, (b) mimotope selection and their computer-aid alignments, with each other and with the primary structure of the protein, and (c) analysis of the epitopes on the computer-algorithm-predicted protein secondary structure. Three epitopes are mapped and investigated in detail, and based on the results, 20 other sites are proposed to contain epitopes, 4 linear and 16 conformational (helical). Perspectives of the use of the strategy in immuno-structural studies of fibrous proteins are discussed.

The human proteomics initiative

U. Hinz, M.-C. Blatter, S. Braconi Quintaje, D. Coral, A. Estreicher, L. Famiglietti, N. Farriol-Mathis, S. Ferro, R. Gatto, A. Gos, N. Gruaz-Gumowski, C. Hulo, J. James, S. Jimenez, S. Poux, S. Reynaud, B. Roechert, A. Stutz, S. Sundaram, and **A. Bairoch**

Swiss Institute of Bioinformatics (SIB), Michel-Servet 1, CH-1211 Geneva, Switzerland

Understanding biological processes requires the integration of sequence data with genetic information, physiological and biochemical data. The Swiss-Prot protein knowledgebase aims to integrate a maximum of information, avoid sequence redundancy and provide our users with reliable, high quality annotation and with cross-references to numerous other databases. It puts emphasis on experimentally verified evidence derived from the literature, databases and contacts with authors. Information from well-characterized proteins can then be used to extrapolate the possible roles of new and uncharacterized proteins.

The Human Proteomics Initiative (HPI) is a major Swiss-Prot project aiming to annotate all known human proteins and their mammalian orthologs. This means providing, for each known protein, a wealth of information that includes the description of its function, domain structure, 3-D structure, interactions with other proteins, information about its expression, etc. Human beings make a huge number of different proteins from a limited number of genes. Representing this diversity is very important and thus we put special emphasis on the annotation of sequence polymorphisms in health and disease, isoforms produced by alternative splicing and on post-translational modifications (PTMs).

Release 41.5 (April 2003) contains about 12,400 annotated human sequences, representing ca. 9,350 different genes. These entries are associated with ca. 24,000 literature references; 23,200 experimental or predicted PTMs, 3,100 splice variants and 15,500 polymorphisms (the majority of which are linked with disease states).

Molecular modeling of gelsolin interactions with its conformational regulators – calcium ions, phosphoinositol 4,5-bisphosphate and caspase-3

Inta Liepina[1], **Paul Janmey**[2], **Cezary Czaplewski**[3], and **Adam Liwo**[3]

[1] Latvian Institute of Organic Synthesis, Aizkraukles str. 21, Riga, LV1006, Latvia

[2] Institute for Medicine and Engineering, University of Pennsylvania, Philadelphia, PA 19104, USA

[3] Faculty of Chemistry, University of Gdansk, 80-952 Gdansk, Poland

Gelsolin is a calcium-activated and phosphatidylinositol 4,5-bisphosphate (PIP2) inhibited actin binding and severing protein, which through cleavage by caspase-3 is also involved in apoptosis. All mechanisms of gelsolin action are based on the conformational changes of the protein.

Molecular modeling of gelsolin interaction with its conformational regulators was performed using molecular dynamics, AMBER 5.0 force field.

Calcium influence on the conformation of the most important gelsolin domain S2 was investigated by molecular dynamics simulations. The results suggest that calcium ions stabilize gelsolin second domain S2.

Gelsolin interaction with phosphoinositol 4,5-bisphosphate lipid was modeled by the peptides G150-169 and G135-142 derived from gelsolin sites interacting with phosphoinositol 4,5-bisphosphate. It was found that binding gelsolin peptides G150-169 and G135-142 to phosphoinositol 4,5-bisphosphate lipid has both hydrophobic and electrostatic nature. It can be supposed that binding gelsolin to phosphoinositol 4,5-bisphosphate is also driven by both hydrophobic and electrostatic forces.

Caspase cleaved N-half of gelsolin, comprising domains S1–S3 was subjected to molecular dynamics simulation. After 2227 ps of molecular dynamics run the three domain structure was still stabile; only slight opening of the structure was observed. Domain S3 moved away from domains S1 and S2. The β-sheet between residues 117–122 of domain S2 and residues 323–328 of domain S3 had a tendency to be disrupted making domain S3 more flexible. After 3770 ps of molecular dynamics run domain S3 partly lost its α-helical and β-sheet structure, but still had two hydrogen bonding contact with domain S1. These results suggest that biggest conformational changes are likely to occur on S1–S3 binding with actin.

An unusual cyclin-dependent kinase: activation mechanism and pharmacological inhibition of CDK5

Marina Mapelli, Lucia Massimiliano, and **Andrea Musacchio**

European Institute of Oncology, Via Ripamonti 435, 20141 Milan, Italy

CDK5 is a member of the small serine/threonine cyclin-dependent kinase (CDK) family. Despite having 60% sequence identity with CDK2 and CDC2, CDK5 is not implicated in cell-cycle coordination. It was shown to play a crucial role in neuronal migration in the developing

central nervous system as well as in the regulation of the cytoskeleton, axon guidance, synaptic function, and dopamine signaling. Like the other CDKs, CDK5 alone shows no enzymatic activity and requires binding to the regulatory subunit p35 for activation.

Recent evidence links CDK5 to the non-receptor tyrosine kinase c-Abl signaling pathway. The c-Abl interactor protein Cables forms a tight complex with CDK5 via its putative cyclin-like box fold. Intriguingly, the phosphorylation of CDK5-p35 by c-Abl at Tyr15 enhances the activity of CDK5 in developing neurons, while the corresponding Tyr15 phosphorylation has an inhibitory effect on other CDKs. We demonstrated that Cables is an *in vitro* substrate for c-Abl but it is not required for the phosphorylation of the CDK5-p35 complex. Our working hypothesis is that Cables competes with p35 for the binding to CDK5 and activates the kinase on a different set of substrates. We have reconstituted the CDK5-Cables complex from co-expression in insect cells and its crystallization is currently underway.

The implication of Tyr15 phosphorylation on inhibition of CDK5 kinase activity by several ATP-analogue moieties will also be discussed.

Proteomic analysis of plant pathogen interactions

Benedetta Mattei

Dipartimento di Biologia Vegetale, Università di Roma ''La Sapienza'', Roma, Italy

Plants are continually exposed to a vast array of potential pathogens; in many cases, they resist attack by blocking fungal development soon after penetration. Since plants lack a circulatory system and antibodies, they have evolved a defense strategy by which each cell is capable of defending itself by means of a combination of constitutive mechanisms and induced responses. The ability of plants to defend themselves against the majority of potential pathogens depends on sensitive perception mechanisms that recognize microbial invaders and subsequently activate defense responses. It is generally accepted that recognition is mediated by signal molecules (elicitors) produced by the pathogen and complementary plant ''receptor'' molecules. Signals and receptors initiate a signal transduction pathway leading to the activation of various defense responses. Although genetic approaches have shown that the various resistance genes activate multiple signal transduction pathways and that common defense responses can be activated via independent pathways, they have been far less successful in identifying the signaling components involved. Proteomic approaches have been used to complement existing genetic studies to elucidate the complex patterns of signaling after the recognition of microbial elicitors and to identify proteins that are phosphorylated rapidly in the response of plant cells to microbial elicitors.

Heavy metal-induced peptides and proteins in the aquatic fungus *Heliscus lugdunensis* Sacc. et Therry

J. Miersch[1], **M. Friedenberger**[1], **R. Augustin**[1], **S. Wohlfromm**[1], **M. Rother**[1], **S. Menge**[1], **A. Schierhorn**[2], **P. Rücknagel**[2], and **G.-J. Krauß**[1]

[1] Martin-Luther-University Halle-Wittenberg, Department of Biochemistry/Biotechnology, Institute of Biochemistry, 06120 Halle, Germany
[2] Max-Planck-Research Unit for Enzymology of Protein Folding, 06120 Halle, Germany

Fungi have several important features that determine how they deal with metal pollution in the environment. Aquatic fungi are dominant decomposers of vascular plant remains in streams. They condition plant litter for further utilization by bacteria and animals.

Recently we investigated the effect of heavy metals on aquatic fungi. We found that *Heliscus lugdunensis* increased synthesis of total buffer-soluble thiols and glutathione in the presence of cadmium, but not of copper and zinc. In order to examine furthermore the cellular stress response to cadmium proteine profiles of the aquatic hyphomycete *Heliscus lugdunensis* were characterized. Cadmium-induced proteins were detected after addition of ^{35}S-cysteine and separation by two-dimensional sodium dodecylsulfate polyacrylamide gel electrophoresis. New proteins were found in the range of molecular masses at 5 to 15 kDa which have not previously been described. After purification of proteins using acetone precipitation, size exclusion chromatography, affinity chromatography and ultrafiltration clear differences between control and cadmium-induced samples were shown. Some of these proteins react with antibodies against metallothionein. The proof of protein spots against a heat shock antibody of the 17 kDa family failed, however. Labelling of the purified protein fractions (8–16 kDa) with ^{14}C-jodacetamide resulted in distinct radioactive spots. Three of them were sequenced N-terminal yielding ubiquitine, an unknown protein containing a threonine-reach fragment and a protein with acidic amino acid-reach fragment. The latter protein may play a role in cadmium detoxification.

Proteomic analysis of the human spliceosome

Juri Rappsilber[1,2], **Ursula Ryder**[3], **Angus Lamond**[3], and **Matthias Mann**[2]

[1] FIRC Institute for Molecular Oncology (IFOM), Milan, Italy
[2] Center for Experimental BioInformatics, University of Southern Denmark, Odense, Denmark
[3] Department of Biochemistry, University of Dundee, Dundee, UK

The spliceosome is a multi-protein-RNA complex responsible for the excision of intronic sequences from pre-mRNAs in eukaryotic cells. The resulting mRNA is used as the template for protein synthesis by the ribosome. In a previous proteomic study of the human spliceosome we identified 42 spliceosome associated factors, including 19 novel ones. Using enhanced mass spectrometric tools and improved databases, we now report identification of nearly 300 proteins that co-purify with splicing complexes assembled on two separate pre-mRNAs. All known essential human splicing factors were found, while 96 novel proteins were identified, of which 55 contain domains directly linking them to functions in splicing/RNA processing. 20 proteins related to transcription were also detected, suggesting a direct connection between this process and splicing. This investigation provides the most detailed inventory of human spliceosome associated factors to date and the data suggest a number of interesting links coordinating splicing with other steps in the gene expression pathway. The mass spectrometric data acquired separately for the two different pre-mRNAs allows for the first time quantitative assessment of the differences in the entire protein composition of two spliceosomal machineries.

Structural studies on NAD biosynthesis and regulation

Silvia Garavaglia, Franca Rossi, and **Menico Rizzi**

DISCAFF-University of Piemonte Orientale ''A. Avogradro'', Via Bovio 6, 28100 Novara, Italy

Once thought to function only as a guardian for the maintenance of the redox potential of the cell, NAD(P) is today considered as a key molecule for several others biochemical processes, including DNA repair and recombination, calcium-mediated signalling, ageing and modulation of transcription. The vital role played by the pyridine dinucleotide renders its homeostasis an attractive source of new targets for the design of novel antibacterial and antineoplastic agents. We started a long-term project aimed at the structural analysis of enzymes involved in NAD biosynthesis and regulation. NAD synthetase and NMN adenylyltransferase, two key enzymes for NAD biosynthesis in all living organisms, have been structurally characterized in different states. The structural analysis revealed both a remarkable conservation of the three-dimensional architecture and a

common strategy for catalysis, despite the ATP binding site is completely different in the two enzymes. Within the framework of our structural studies on the regulation of NAD homeostasis in bacteria, both at transcriptional level and through allosteric control, we are presently carrying out the structure determination of *E. coli* NadR, a NAD-dependent repressor for the transcription of genes involved in NAD biosynthesis, in complex with DNA. Moreover, we discovered that *B. subtilis* NAD kinase, the obligatory enzyme for NADP synthesis, showed a marked positive cooperativity for ATP and was strongly activated by quinolinic acid, the central metabolite in *de novo* NAD biosynthesis, suggesting a major regulatory role for the enzyme in NADP biosynthesis. Crystals of *Mycobacterium tuberculosis* NAD kinase have been obtained in our laboratory and the ongoing structure determination will reveal the molecular basis for the observed allosteric regulation.

Chemical communication in insects: structural analysis

V. Campanacci, A. Lartigue, C. Cambillau, and **M. Tegoni**

Architecture et Fonction de Macromolécules Biologiques, UMR 6098-CNRS, Université Aix-Marseille I et II, 31, Chemin Joseph Aiguier, 13402 Marseille Cedex 20, France

Olfaction is a primordial sense in insects as the interactions with other individuals (social and mating) and with the environment depend on it, thus sensory organs and in particular antennae (sensillae) are very developed in insects, and especially in Lepidoptera (night moths). Small and soluble proteins, likely involved in the olfactory process, were found in high concentration (10 mM) first in sensillae (Pheromone Binding Proteins, PBPs) and later in sensory organs (Chemo Sensory Proteins, CSPs). PBPs ($\sim$150 aminoacids, 3 disulfide bridges) have been shown to bind pheromones and proposed to carry the hydrophobic pheromone through the antennal lymph to the receptors, participating thus in the peri-receptor events of signal transduction. CSPs ($\sim$115 aminoacids, 2 disulfide bridges) were found in antennae, proboscis, labrum and tarsi of a wide variety of insect species (cockroach, phasmida, locust, moth). They are proposed to be involved in olfaction or taste although their physiological ligands are still unknown. No sequence homology between PBPs and CSPs has been evidenced.

We cloned and expressed in the periplasm of *E. coli* the soluble form of these proteins from the moth *Mamestra brassicae* or the cockroach *Leucophaea maderae*. We then characterized by fluorescence their binding properties towards the physiological or surrogate ligands, and resolved their structure by X-ray crystallography, in the unbound or bound form.

These proteins show a new fold composed of 6 α-helices, delimiting a cavity in which the ligand or the fluorescence reporter is embedded. The high flexibility of these proteins is likely to be related to the mechanism of signal transduction.

Novel sample preparation techniques for enhanced protein identification by mass spectrometry

Alistair Wallace[1], **Jim Langridge**[1], **Emmanuelle Claude**[1], **Richard Tyldesley**[1], **Dominic Gostick**[1], **Robert Bateman**[1], **Jeff Brown**[1], **Kevin Howes**[1], **Ed Bouvier**[2], **John L. Gebler**[2], **John Peter Lee**[2], **Simon Gaskell**[3], **Ian Beningfield**[3], and **Darryl Pappin**[4]

[1] Waters Corporation, Manchester, UK
[2] Waters Corporation, Millford, MA, USA
[3] The Michael Barber Centre for MS, UMIST, Manchester, UK
[4] Imperial College, London, UK

Mass spectrometry has become an essential tool for the analysis of biopolymers (proteins, peptides, and oligonucleotides). It is well known that the sensitivity and quality of analysis is highly dependent on preparation and purity of the sample. In particular, salts and surfactants commonly employed in the isolation of biopolymers dramatically affect the quality of the resulting mass spectra due to ion suppression or adduct formation. Thus, sample manipulation to remove interfering materials is often necessary. Methods such as precipitation, solid phase extraction, or off-line chromatography are laborious, costly, and often result in sample loss.

The first part of this presentation will discuss two new emerging technologies, which simplify sample preparation for analysis. First described will be the use of a modified MALDI plate that facilitates the concentration and desalting of peptide and protein mixtures directly on the plate. The plate is robust, reproducible, and makes possible detection limits in the sub-femtomolar range. Comparison with other common methods used for the concentration and desalting of peptide samples will be shown. A second topic will discuss the use of a novel reagent, RapiGest^{TM}, to aid in the endopeptidase in-solution digestion of proteins. RapiGest^{TM} facilitates rapid digestion (<30 min) of soluble proteins and enables the digestion of hydrophobic or proteolytic resistant proteins. In addition, RapiGest^{TM} is easily removed by acid treatment and does not interfere with MALDI or LC\ESI-MS analysis.

MALDI-TOF-MS provides a peptide mass fingerprint of the protein digests and allows the rapid and accurate identification of the parent protein by comparison to a databank. In the case of low abundance proteins, only a few peptides may be detected and databank searching can lead to an ambiguous result. One of the problems is that arginine-containing peptides dominate MALDI Tof spectra [Krause et al., *Anal. Chem.* 1999; **71**: 4160]. Lysine-terminating peptides in particular are less likely to be detected since the lysine sidechains are low in basic nature and less likely to be ionised. In peptide mass fingerprinting, if the number of peptides detected is small or if the resulting sequence coverage is poor, it is advantageous to be able to include even short pieces of sequence information to provide added specificity in databank searching. This information can be obtained when the MALDI ionisation technique is coupled with a high performance MS/MS mass spectrometer (MALDI Q-Tof). The peptides are singly charged due to the MALDI ionization process and this often results in incomplete sequence information from the MS/MS data. It would be advantageous in this case if the fragmentation efficiency of these peptides could be increased to recover this information.

The second part of this presentation will discuss different methods of chemically modifying peptides in order to either increase the overall quality of MALDI-TOF-MS spectra used for peptide mass fingerprinting, or increase their fragmentation efficiency and therefore the amount of sequence information obtained using MALDI-MS/MS.

Molecular cloning and recombinant expression of antimicrobial peptides from house fly, *Musca domestics*

Jin-Xing Wang, Xiao-Fan Zhao, Lai-Cheng Wang, Lei Li, and **Xin-Jun Du**

School of Life Sciences, Shandong University, Jinan, Shandong, 250100, P.R. China

Two antimicrobial peptide genes, cecropin and defensin, were cloned by PCR from the cDNA library of houseflies, *Musca domestics*, which had been challenged by *Escherichia coli* and *Staphylococcus aureus.* The full length of the cecropin cDNA was 403 base pairs and the open reading frame of the cDNA encoded a 63 amino acide procecropin. The mature peptide contains 40 amino acids. The sequence identity with other cecropin was between 80% to 97%. Another gene was a novel full-length 430 base pairs cDNA of an insect defensin. Sequence analysis revealed that the open reading frame of the cDNA encoded a 92-amino acid peptide, which contained an NH_2-terminal signal sequence (1$\sim$22) followed by a propeptide and the mature peptide (53$\sim$92). The sequence

identity with other insect defensin is between 51% and 73%. The mature peptide, with a predicted molecular weight of 4.0 kDa, and pI of 8.69, has 1 negative charged amino acid and 4 positive ones. Two cDNAs encoding cecropin and defensin were cloned into the pGEX-4T1 and the secretive expression vector pPIC9K, and transferred into *E. coli* BL21 and the special strain KM71 of *Pichia pastoria* respectively. The positive clones screened by phenotype were induced by IPTG and methanol respectively. Recombinant cecropin and defensin were expressed in *E. coli* and methylotrophic yeast successfully. The recombinant cecropin and defensin were confirmed by amino acid sequencing. Antimicrobial activity using *Escherichia coli* and *Staphylococcus aureus* showed that the recombinant antimicrobial peptides expressed in yeast had the activity against the two bacteria.

Proton-pumping mechanism of bovine heart cytochrome *c* oxidase

Hideo Shimada[1], **Kunitoshi Shimokata**[1], **Yukie Katayama**[1], **Kazumasa Muramoto**[2], **Hiroshi Aoyama**[3], **Masao Mochizuki**[2], **Kyoko Shinzawa-Itoh**[2], **Eiki Yamashita**[4], **Min Yao**[4], **Tomitake Tsukihara**[4], **Yuzuru Ishimura**[1], and **Shinya Yoshikawa**[2]

[1] Department of Biochemistry, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan
[2] Department of Life Science, Himeji Institute of Technology and CREST, Japan Science and Technology Corporation (JST), Kamigohri Akoh, Hyogo 678-1297, Japan
[3] RIKEN Harima Institute, Mikazuki Sayo, Hyogo 679-5148, Japan
[4] Institute for Protein Research, Osaka University, 3-2 Yamada-oka, Suita 565-0871, Japan

Cytochrome *c* oxidase reduces dioxygen (O_2) to water coupled with proton pumping as the key process in aerobic cell respiration. Its mechanism has been yet to be elucidated. An aspartate residue (Asp51) located near the enzyme surface, and conserved in the animal enzymes undergoes a redox-coupled X-ray structural change, suggesting redox-driven proton pump involving the residue. However, neither functional (or mutational) evidence for its involvement in the proton-pumping nor the driving mechanism has been given. Here, we report the asparate/asparagine mutation of the bovine enzyme by establishing the system for constructing a bovine-human hybrid enzyme in human cells. The Asp51Asn mutant enzyme shows no proton pump activity but full O_2 reduction activity. Improved X-ray structures (at 1.8/1.9 Å resolution in the fully oxidized/reduced states) and FTIR results show that oxidation of a low-spin heme (heme a) drives the active proton transport to the aspartate across the enzyme via a water channel and a hydrogen-bond network, located in tandem, from the inside of the mitochondrial innermembrane and that the heme reduction induces proton ejection from Asp51 to the outside of the mitochondrial innermembrane. The improved X-ray structure indicates that two possible proton transfer pathways, for the protons coupled with the electron transfers before and after O–O bond cleavage, respectively.

PrP^{Sc} allotypes profiling in bank voles (*Clethrionomys glareolus*) experimentally infected with scrapie

M. E. Schininà [1], **C. Cartoni**[2], **R. Nonno**[2], **G. Vaccari**[2], **M. Di Bari**[2], **M. Conte**[2], **F. Cardone**[3], and **U. Agrimi**[2]

[1] Department of Biochemical Sciences and Centro di Eccellenza BEMM, Università la Sapienza, Rome, Italy
[2] Istituto Superiore di Sanità, Laboratory of Veterinary Medicine, Rome, Italy
[3] Istituto Superiore di Sanità, Laboratory of Virology, Rome, Italy

Scrapie of sheep belongs to prion diseases, a group of fatal neurodegenerative pathologies characterized by the accumulation in the central nervous system of the amyloid protein PrP^{Sc}, which derives from conformational modifications of a cellular precursor called PrP^{C}. Epidemiological and experimental studies have shown that in species where the PrP gene is polymorphic, the genotype composition is an important factor in conditioning the development of prion diseases. The identification of PrP^{Sc} allotypes which accumulate in the brain of heterozygous affected subjects is valuable to investigate whether these polymorphisms are critical for the pathological conversion of PrP^{C}. Analyses are complicated by the insolubility of PrP^{Sc} extracted from brain, but this could be obviated by extensive digestion of extracted fractions and analysis of peptide fragments composition. Recently, the development of an optimised protocol of liquid chromatography-mass spectrometry, has led to maps of PrP peptides in digested fractions with a low PrP^{Sc}/contaminants ratio. We successfully applied this sensitive approach to the analysis of PrP^{Sc} accumulation in the brain of experimentally scrapie-infected PrP-heterozygous bank voles (*Clethrionomys glareolus*), a rodent model in which TSEs could be experimentally transmitted in a very fast and efficient way. Our preliminary results clearly demonstrate in bank vole the accumulation of the two PrP allotypes, thus indicating that they can both undergo to pathological conversion. Additional studies should be carried out to investigate the relative amounts of the two allotypes accumulating in the brain and to study the allotype profile of prion diseases caused by different prion strains.

Selenocysteine

Inhibition of the selenoprotein thioredoxin reductase by electrophilic prostaglandins

Pamela B. Cassidy[1], **Philip J. Moos**[2], **Kornelia Edes**[2], and **F. A. Fitzpatrick**[1]

[1] Huntsman Cancer Institute, Departments of Medicinal Chemistry and [2] Oncological Sciences, University of Utah Salt Lake City, UT 84112, USA

We have found that electrophilic cyclopentenone prostaglandins of the A and J series bind to a number of cellular proteins. Using biotinylated prostaglandins, we identified one of these proteins as thioredoxin reductase (TrxR). We have evaluated the interactions of purified TrxR with cyclopentenone prostaglandins and report that the inhibition of TrxR requires NADPH and is irreversible. The site of modification of TrxR by 15-deoxy-Δ-12,14-PGJ_2 has been determined. We also found that TrxR-dependent phenomenon such as p53 function (apoptosis, DNA binding), and the activation of hypoxia-inducible factor were disrupted in prostaglandin-treated cells. Our results provide a framework for understanding how prostaglandins and other chemically related endogenous and exogenous electrophiles participate in carcinogenisis, and how chronic inflammation might heighten the risk for cancer.

Bioactivation and biological activity of selenocysteine Se-conjugates

Jan N. M. Commandeur

Section Molecular Toxicology, Leiden/Amsterdam Center for Drug Research, Vrije Universiteit, 1081 HV Amsterdam, The Netherlands

Synthetic organoselenium compounds have been shown to possess interesting pharmacological properties. Dependent on their chemical

structures, these activities may be antioxidant activity, anti-inflammatory activity, chemopreventive and antitumor activity and chemoprotective activity. The biological activity of organoselenium compounds is believed to be mediated by several metabolites, such as selenoxides, selenols and/or selenenic acids. These metabolites can influence the activity of proteins by interfering with their redox-status, by covalent binding to regulatory thiol or selenol groups, or by removal of zinc from inhibitory zinc-specific enzymic sites (Ganther, *Carcinogenesis* 20, 1657, 1999).

Recently, Se-conjugates of selenocysteine (SeCys-conjugates) were shown to possess potent chemopreventive activity in several animal-models for chemical carcinogenesis. The chemopreventive activity was much higher than that of corresponding sulfur-analogs, indicative for a crucial role of the selenium-atom. Several biochemical mechanisms of action may contribute to the protective activities of SeCys-conjugates, including induction of phase II-enzymes, inhibition of bioactivating enzymes, induction of apoptosis in transformed cells and anti-angiogenesis. The selenium-species actually responsible for these effects is not yet identified.

In this presentation, the bioactivation mechanisms which may be involved in the biological activities of selenocysteine Se-conjugates will be discussed.

The mechanism of mammalian thioredoxin reductase studied by semi-synthesis

Robert Hondal

Department of Biochemistry, The University of Vermont, USA

Mammalian thioredoxin reductases are homodimeric flavoproteins that catalyze the reduction of thioredoxin using NADPH as a cofactor. The mammalian enzymes contain the unusual amino acid selenocysteine, which is encoded by the DNA with a UGA codon. The UGA codon is normally a stop codon, but in some cases can be recoded as a sense codon for selenocysteine. The unusual codon requirement for proteins containing selenocysteine presents a barrier to heterologous expression in prokaryotes such as *E. coli*. One approach to producing selenium containing enzymes is semi-synthesis. Thioredoxin reductase can be produced as an intein fusion protein. Thiolytic cleavage of the fusion protein results in a thioester-tagged thioredoxin reductase. The thioester group is used as a site of chemoselective ligation to ligate a tripeptide containing the C-terminal peptide containing selenocysteine. A second method of producing a semi-synthetic enzyme is peptide complementation. Both methods will be discussed in relation to studying the enzymatic reaction mechanism.

Selenazolidine prodrugs of selenocysteine as cancer chemopreventive agents

Jeanette Roberts, Xie Yang, Liang Li, Wael El-Sayed, Pamela Cassidy, and **Megan Short**

Department of Medicinal Chemistry, University of Utah, Salt Lake City, UT, USA

Selenium is of growing importance in human health beyond its well-recognized role as a micronutrient. For example, selenium has exhibited exciting activity as a cancer chemopreventive agent against disease in several organs, caused by a variety of carcinogens. Selenium is also known for its toxicity, however, making the development of clinically valuable agents a distinct challenge that must be accomplished with extreme care and creativity. Many selenium-containing compounds are being studied and used as selenium supplements (sodium selenite, selenomethionine, Se-methylselenocysteine, etc.). Selenocysteine may represent a biochemically superior form in which to provide selenium due to the differential metabolic processing of the various forms. However, the free amino acid is chemically unstable and difficult to handle. Therefore, we have designed and synthesized selenazolidine "prodrugs" of selenocysteine as chemically superior forms of the amino acid. Prototype selenazolidine prodrugs have been studied for their basic biochemical properties, as well as their cancer chemopreventive activity against a tobacco-derived lung carcinogen in the A/J mouse model.

Selenoproteins and glutathione peroxidase mimics: defense against hydroperoxides and peroxynitrite

H. Sies[1], **G. Mugesh**[2], and **K. Hideg**[3]

[1] Institut für Biochemie und Molekularbiologie I, Heinrich-Heine-Universität Düsseldorf, Postfach 101007, D-40001 Düsseldorf, Germany
[2] Department of Inorganic & Physical Chemistry, Indian Institute of Science Bangalore 560 012, India
[3] Institute of Organic and Medicinal Chemistry, University of Pécs, P. O. Box 99, H-7602 Pécs, Hungary

Micronutrients such as selenium play a role in defense against hydroperoxides and peroxynitrite, compounds utilized by the organism in inflammatory responses against bacteria and other invading organisms. Healthy bystander cells may become exposed to peroxynitrite generated by inflammatory cells, and the intracellular components of these cells themselves require protection as well. Selenium is incorporated into proteins as selenocysteine or as selenomethionine, and both these aminoacids can serve to reduce peroxynitrite to nitrite. Selenoenzymes catalyzing the reduction of peroxynitrite include GSH peroxidase, thioredoxin reductase and selenoprotein P. The latter may be well-suited to protect the surface of the endothelial cells, because selenoprotein P can bind to heparan sulfates. During inflammatory conditions there is an acidification, and due to the electrostatic interaction of selenoprotein P with the surface proteoglycans there is an increased localization of the protein to the cellular surface.

A number of selenoorganic compounds has been synthesized to mimic selenocysteine-related actions. One compound, ebselen, has been utilized in various systems of inflammatory models *in vitro* as well as *in vivo*, and there have been successful human clinical trials using ebselen in the therapy of cerebral ischemia, *e.g.* stroke. We have recently synthesized ebselen-related compounds with added pyrroline structures, augmenting the activity as glutathione peroxidase mimics. Thus, dual-activity compounds might be of interest in pursuing further therapeutic strategies.

Selenium delivery proteins and selenophosphate biosynthesis

Thressa C. Stadtman

Laboratory of Biochemistry, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892-8012, USA

Selenophosphate appears to be the universal donor of selenium for specific selenoenzyme biosynthesis. We are finding that the high levels of toxic selenide used as substrate for selenophosphate biosynthesis *in vitro* can be avoided by use of selenium transferases or selenium delivery proteins. Those enzyme systems that have been shown to function *in vivo* include selenocysteine lyases that deliver an atomic form of selenium directly to selenophosphate synthetase. In mammals, selenocysteine can be derived from selenoproteins present in ingested foods. Complex culture media that contain yeast, plant, or animal protein extracts can serve as selenocysteine source for various prokaryotes. Also, certain prokaryotes, such as *Escherichia coli*, can synthesize selenocysteine via the cysteine biosynthetic pathway. Direct mobilization of selenium from inorganic sources can be accomplished by various selenium-binding proteins related to the rhodanese enzyme family. In the case of sulfur mobilization, rhodanese utilizes thiosulfate as substrate and an enzyme bound persulfide is formed as the intermediate. Selenite reduced by

thiols, such as glutathione, to RS-Se-SR can be utilized by enzymes of this class to form the corresponding bound perselenide intermediate that serves as substrate for selenophosphate biosynthesis. The identification and characterization of these various types of selenium delivery proteins from the anaerobic methane producing organism, *Methanococcus vannielii*, will be described. *Methanococcus vannielii* was selected for study because it synthesizes several essential selenoenzymes and is a rich source of the required precursors.

Unraveling new functions of selenium catalysis in peroxidases

Fulvio Ursini

Department of Biological Chemistry, University of Padova, viale G. Colombo 3, I-35121, Padova, Italy

PHGPx is a seleno-peroxidase catalysing the reduction of lipid hydroperoxides, and thus acknowledged as an antioxidant enzyme. However, the reaction catalysed by PHGPx has also been seen as a regulatory factor in signalling cascades and apoptosis and as the core element leading to the formation of mitochondrial capsule in mature mammalian spermatozoa, where proteins are cross-linked in a network disulfide bonds. In this process, following the oxidation of catalytic Sec, a selena-disulfide is formed with a suitable protein Cys, as reducing substrate, alternative to GSH. It is then assumed that thiol-disulfide exchange reactions finalize the production of the complex cross-linked network. The formation of initial PHGPx polymers has been elucidated using an LC/ESI-MS/MS approach. Analysis of MS spectra revealed that the process is due to a selective reaction of Sec-46 with Cys-148′ resulting in linear polymers. FT docking of PHGPx molecules confirmed the specificity of this reaction. This step actually full fits the requirements for formation of the postulated second intermediate of the catalytic cycle of Se dependent thiol-peroxidases. This was confirmed by showing that a Se-glutathionylated enzyme can be produced in the presence of GSH and, remarkably, also in the presence of GSSG. According to molecular models, specific binding of both GSH and of GSSG to the active site of PHGPx is promoted by electrostatic attraction of Lys-48 and Lys-125. This evidence indicates that this step of the peroxidatic catalytic cycle is reversible and that the steady-state of concentration of the selena-disulfide -driving protein thiol oxidation- must depend on the GSH/GSSG ratio, i.e. on the redox potential of the cell. It is concluded that the same catalytic principle drives the seemingly diverse actions of selenium catalysis of PHGPx, depending on redox conditions, i.e. hydroperoxide reduction, GSSG reduction, S-derivatization and protein thiol oxidation, including self-incorporation of PHGPx into biological structures.

Sport and Exercise

The future of antidoping tests between forensic analysis and toxicological evaluations: from markers of exposure to markers of effects?

Francesco Botrè

Laboratorio Antidoping, Federazione Medico Sportiva Italiana and Department CGMIA, University ''La Sapienza'', Rome, Italy

The use of performance-enhancing substances in sport threatens not only the meaning and the ethical value of the sport itself, but also the health of the athletes. So far, the fight against doping in sport has been carried out mostly by selected antidoping laboratories, accreditated by the International Olympic Committee and by the World Anti-Doping Agency. Despite the increasing complexity of the analytical protocols for the detection of doping agents, some key apsects of anti-doping policies are not yet fully managed by the activity of the testing laboratories. More specifically, laboratory tests do not allow to evaluate the possible toxicological risks associated with the abuse of drugs, either licit or illicit, by sportsmen, especially concerning the ''unknown'' risks deriving from the combination of two or more doping agents. Particularly, the most ''advanced'' forms of doping involve the administration of recombinant proteic and/or glycoproteic hormones whose direct detection in biological fluids is at present possible only for a limited variety of peptides; at the same time, toxicological risks due to their abuse are very high. The approach followed in other fields of toxicological analysis (like, for instance, in food and environmental controls) can reveal very effective also in the field of antidoping control: toxicological methods (primarily among them techniques based on cell toxicity tests, but also those involving inhibition biosensors, simulation studies, and *in silico* methods) could provide extremely useful information to integrate the ''traditional'' analytical results supplied by the antidoping laboratories. Such an integrated approach could allow to increase the depth of field of the doping control activity, focusing not only on the identification of markers of ''exposure'', but also on a more complete assessment of markers of ''effect'' of doping agents.

Acute sleep deprivation, fatigue, immunodepression and glutamine

Linda M. Castell[1], **Emily Barton**[2], **Patrick Hickey**[2], **Anna-Marie Michel**[2], **Rebecca Cardenas**[2], **Laura Sanchez**[2], and **James C. Miller**[2]

[1] Nuffield Department of Anaesthetics, University of Oxford, Oxford OX2 6HE, UK

[2] CASL, Brooks-City Base, San Antonio, TX 78235, USA

Sleep deprivation occurs in shift work, long-haul flights, and hospitalization. Sleep patterns are altered in chronic fatigue syndrome patients, and in athletes with overtraining. Frequent minor infections occur in these individuals: psychoimmunological links have been suggested, also a link between sleep deprivation and immunodepression. The present study investigated one night's sleep loss on immune cell numbers and plasma metabolites, followed by three subsequent nights of normal sleep, and glutamine feeding. Healthy male subjects were studied for four days. Cognitive function tests, sleepiness, mood states and incidence of illness were monitored. Fasting, resting blood samples were taken daily: full blood counts, some plasma metabolites and cytokines were measured. Subjects received glutamine/placebo daily.

Sleepiness significantly increased after the night's sleep deprivation ($p < 0.0001$; at Day 2). Cognitive function was markedly reduced at Day 2. Anger-Hostility decreased at Days 3–4 ($p < 0.042$); Tension-Anxiety was significantly higher at Day 2 ($p < 0.046$). Lymphocyte numbers and eosinophil percentages decreased at Day 3 ($p < 0.071$ and $p < 0.045$). Neutrophil percentage decreased with glutamine feeding at Day 2 ($p < 0.05$). Trends observed in glutamine versus placebo group included: lower lymphocyte numbers at Day 3; lower monocyte numbers and percentages throughout the study; platelet numbers higher at Days 3 & 4. CD4/CD8 cell ratio decreased at Day 3 in both groups. In eight out of fourteen subjects, serum leptin decreased (by $24 \pm 4\%$) at Day 2. Leptin is linked with sleep disturbance, exercise fatigue and cognition.

One night's sleep deprivation had a measurable effect on some immune cells, plasma metabolites and psychological tests. Our future

studies will measure cytokine production in conjunction with neutrophil function.

Amino acid concentrations in blood and muscle following bovine colostrum supplementation

Antti Mero[1], **Hannu Pitkänen**[1,2], **Tarja Nykänen**[1], **Juha Knuutinen**[3], **Kaisa Lahti**[3], **Olavi Keinänen**[4], **Markku Alen**[5], **Simo Rasi**[6], and **Juhani Leppäluoto**[6]

[1] Department of Biology of Physical Activity, University of Jyväskylä, Jyväskylä, Finland
[2] Kankaanpää Rehabilitation Centre, Kankaanpää, Finland
[3] Department of Chemistry, University of Jyväskylä, Jyväskylä, Finland
[4] Clinical Physiology, Central Finland's Central Hospital, Jyväskylä, Finland
[5] Peurunka-Medical Rehabilitation Center, Laukaa, Finland
[6] Department of Physiology, University of Oulu, Oulu, Finland

The responses of blood serum and muscle amino acid concentrations to bovine colostrum supplementation were analyzed in physically active adult men. As subjects there were six men in the rest (R) group and six men in the exercise (E) group. Both groups carried out twice a 2-week experiment randomly assigned in a double-blind fashion with either placebo (PLA; consuming daily 20 g maltodextrin) or bovine colostrum (COL; consuming daily 20 g colostrum supplement) treatment. On the test day after treatment the measurements were carried out in fasting conditions in both groups. The R group rested quietly but the E group carried out a hypertrofic strength training session (STS) of 50 minutes for lower extremities. Blood samples from a femoral vein (FV) and a femoral artery (FA) for analysis (RPHPLC) of free amino acid concentrations were taken at 60 and 195 min after STS or respective time period at rest. At the same time periods a muscle biopsy from the vastus lateralis muscle was taken for analysis (HPLC) of free amino acid concentrations. The results showed that serum concentration of essential amino acids ($P < 0.05$) and branched chain amino acids ($P < 0.01$) were greater in the COL groups compared with the PLA groups. There were no differences in the amino acid concentration of muscle between the COL and PLA groups. The expected exercise-induced increase in alanine concentration decreased both in serum and muscle. In conclusion, a 2-week supplementation with bovine colostrum increases essential amino acid concentration in serum but not in muscle.

Comparative studies of changes in amino acid profiles after aerobic and anaerobic exercise

Roberta J. Ward[1], **Hannu Pitkanen**[2], **Robert R. Crichton**[1], **Marc Francaux**[3], and **Antti Mero**[2]

[1] Unité de Biochimie, University of Louvain, Louvain-la-Neuve, Belgium
[2] Department of Biology of Physical Activity, University of Jyvaskyla, Jyvaskyla, Finland
[3] Unite d'education physique, University of Louvain, Louvain-la-Neuve, Belgium

There have been numerous studies in the changing profiles of circulating amino acids after exercise although, as yet, there have been no reports of a comparison between the different amino acid profiles after aerobic and anaerobic running exercises. After an endurance event, a Marathon (aerobic exercise), the majority of amino acids decreased significantly by approximately 2 fold. However, after a long run session of 20 second runs with recoveries of 100 seconds until exhaustion on treadmill (anaerobic exercise) both alanine and arginine significantly increased. Taurine, a sulphonated amino acid, increased significantly after both aerobic and anaerobic exercise although methionine showed no significant changes after either exercise. Such results indicate the role played by amino acids during exercise, their degradation in aerobic exercise for possible use in gluconeogenesis, while the increases in alanine in anaerobic exercise indicate the uptake of lactate by the liver by the Cori cycle to yield glucose for utilization by the muscle.

Detection of erythropoietin doping in urine samples – strategies of data interpretation

Günter Gmeiner

Doping Control Laboratory, Austrian Research Centers, Seibersdorf, Austria

The development of a method for the direct detection of the application of recombinant erythropoietin (EPO) in urine samples by Lasne et al. (2000) lead to a break through in the detection of protein doping. The method consists of concentration of the urine by ultrafiltration, separation of the isoforms by isoelectric focussing, double blotting with the use of a highly specific antibody and chemiluminescent detection.

After the implementation of this method by various IOC-accredited doping control labs, strategies for a proper evaluation of the data deriving from the isoelectric patterns of the different epo isoforms have been developed.

This contribution discusses different approaches to standardize and harmonize the interpretation of the patterns. For this purpose a prototipe of an easy-to-use software package, called GASEPO, for the use across doping control laboratories world wide has been developed thus providing for international harmonization of EPO analysis.

Anabolic steroids in sports nutrition: the Austrian experience

Günter Gmeiner

Doping Control Laboratory, Austrian Research Centers, Seibersdorf, Austria

Since the first indications of the presence of anabolic steroids in sports nutrition without any notice on the label, recent investigations in different IOC-accreditated doping control laboratories have shown, that this problem is still pending to be solved. Concentrations of anabolics have been found in a range, where in addition to the risk of providing a positive doping test result, adverse health effects have to be expected.

This contribution compares national and international studies conducted to highlight the scope of the problem and presents the Austrian model of co-operation between sports federations, national anti-doping agencies, sports nutrition manufacturers and accredited laboratories. The model focusses to reduce the risk of an unintentionally positive doping test to a minimum.

Application of LC-MS-MS to the study of the differences between endogenous and recombinant peptides: the glicosylation profile of native and recombinant human erythropoietin

Bruno Casetta[1], **Monica Mazzarino**[2,3], and **Francesco Botrè**[2,4]

[1] Applied Biosystems Italia, Monza, Italy
[2] Laboratorio Antidoping, Federazione Medico Sportiva Italiana, Roma, Italy
[3] Department of Pharmacology and General Physiology, University "La Sapienza", Rome, Italy
[4] Department CGMIA, University "La Sapienza", Rome, Italy

Most methods currently employed for the screening and confirmation of doping agents in human urine are based on analytical techniques used

for the trace analysis of organic compounds in complex matrices: gas chromatography coupled to mass spectrometry is at present the first choice, both as a screening and as a confirmation technique, in all the 30 antidoping laboratories accreditated by the International Olympic Committee (IOC) and by the World AntiDoping Agency (WADA). This approach has revealed very effective to detect the abuse of stimulant, narcotics, steroids, diuretics, beta blockers, beta agonists, local anaesthetics and of a great variety of drugs of abuse and of low molecular weight masking agents. On the other hands, new forms of doping in sport involve the use of peptide hormones produced by the technique of recombinant DNA, including erythropoietin (EPO) and its synthetic derivatives, growth hormone, insulin and insulin-like growth factors. In these cases the contribution of protein chemistry and molecular biology may be envaluable. The most striking example of such an approach is the Lasne protocol, based on the technique of ''double blotting'', currently employed by selected IOC-WADA laboratories for discriminating between native and recombinant EPO. However, since the method is time consuming and expensive, it is presently being employed only as a confirmation protocol, to be applied on those samples failing a preliminary screening, represented by multiparametric hematological evaluations.

We are here presenting some preliminary results obtained by studying the glicosylation profile of EPO by LC-MS-MS. Samples of native and recombinant EPO were preliminary digested with trypsin, pre-purified and assayed by LC-MS-MS (triple quadrupole). Reproducibile results were obtained on different lots of human recombinant EPO, showing at the same time pronounced differences with samples of native human EPO. While it may be not possible to match the current IOC-WADA confirmation criteria for reporting a sample positive for recombinant EPO, the approach here presented may be effectively employed as a screening technique, preliminary to the confirmation by the Lasne protocol, allowing a drastic reduction of the time and costs of operation and, at the same time, eliminating the need for combined blood-urine sampling in testing for EPO and its analogues.

Effect of amino acid supplementation on the urinary steroid profile concentrations

Francesca Rossi[1,2], **Giuseppe De Feo**[2], and **Francesco Botrè**[1,3]

[1] Laboratorio Antidoping, Federazione Medico Sportiva Italiana, Roma, Italy

[2] Department of Pharmacology and General Physiology, University ''La Sapienza'', Rome, Italy

[3] Department CGMIA, University ''La Sapienza'', Rome, Italy

Food supplements, and especially those containing the branched chain amino acids (BCAA) valine, leucine and isoleucine, are widely used by sportsmen, not only at elite level. We have investigated their possible effects on the urinary concentration of testosterone, epitestosterone, androsterone, ethiocolanolone, 11OH-androsterone, 11OH-ethiocolanolone, estrone, estradiol, estriol, to evaluate whether the variations – if any – could led to a misinterpretation of the urinary steroid profile in antidoping tests.

All food supplements used in this study were preliminarily analyzed by a GC-MS procedure to exclude any contamination with substances not indicated in the label.

The urines of 20 male healthy volunteers (age 25–40) moderately trained (1–2.5 hrs $\times$ 3 times/week) and taking food supplements were analyzed prior to, during and after the period of supplementation with (a) minerals (b) vitamins and (c) BCAA. All urinary values were corrected for the specific gravity (normalized for 1.020). Samples with pH > 7 were not considered if the unconjugated/conjugated ratio for testosterone was $>10\%$. Circadian variability was studied for all subjects participating to the study: the analysis of all samples (at least 2 times/months for each subject) randomly collected at 10 am for 4 months.

Urine ample were assayed by a specifically developed procedure: after measurement of pH and specific gravity, 3 ml uf urine were passed on C18 cartridges, the MeOH eluate was hydrolyzed by b-glucuronidase, pre-concentrated under N2 stream, derivatized to form TMS-derivatives, and assayed by GC-MS.

Supplementation with minerals and vitamins did not provoke any alteration of the steroid profile, while supplementation with BCAAs caused a statistically significant reduction of the absolute urinary concentration values of androgenic steroids.

Taurine

The oxidation of the sulfinates, hypotaurine and cysteine sulfinate, by peroxynitrite

Mario Fontana, Donatella Amendola, Silvestro Duprè, and **Laura Pecci**

Dipartimento di Scienze Biochimiche ''A. Rossi Fanelli'', Università degli Studi di Roma ''La Sapienza'', Piazzale Aldo Moro 5, I-00185, Roma, Italy

Hypotaurine and cysteine sulfinate are recognized as key intermediates in the metabolic pathway leading from cysteine to taurine. The oxidation of the sulfinic group to the respective sulfonate is a crucial point for the generation of taurine in mammalian tissues. However, the mechanism of the oxidative reaction of the sulfinic group is not yet clearly defined. In the last years, peroxynitrite, which is formed by the reaction between $^{\bullet}NO$ and $O_2^{\bullet -}$, has been receiving increasing attention as a mediator of human diseases. Peroxynitrite is a potent oxidant able to oxidize and nitrate a variety of biological molecules. Peroxynitrite can perform either two- or one-electron oxidative reactions. The reaction of peroxynitrite with higher oxidation state of biological sulfur such as sulfinates has not been explored up to now. In our study, we present and discuss data on the interaction of peroxynitrite with the sulfinates, hypotaurine and cysteine sulfinate, showing that these compounds are oxidized by peroxynitrite into the corresponding sulfonates, taurine and cysteate, respectively. Our data indicate that the oxidation of the sulfinic group of hypotaurine and cysteine sulfinate is performed essentially by one-electron mechanism with the involvement of transient radical intermediates, such as sulfonyl radical ($RSO_2^{\bullet}$). Peroxynitrite can therefore be included into the not specific biological oxidant able to accomplish the last step of the metabolic production of taurine. The possible formation of sulfur-centered radical in the conversion of sulfinate to sulfonate by peroxynitrite raises also the question about the metabolic fate and/or the pathophysiological significance of these transient species.

Taurine and early events in the life

F. Franconi[1], **A. Fortuna**[2], **G. Galietta**[2], **G. Trombetta**[3,2], and **A. Loizzo**[2]

[1] Department of Pharmacology and Center of Biotechnology and Biodiversity of University of Sassari [2] ISS, Roma [3] ASL8 Regione Calabria, Italy

Taurine is a non-proteic sulphur aminoacid, which has involved in the development. Recently, it has been evidenced that mother with low

taurine plasma levels generate offspring with alterations in central nervous system and endocrine pancreas. Interestingly, these alterations are also present in adult life and in the second generations (Aerts L, Van Assche FA (2002) J Perinat Med 30: 281). Recently it was shown that in mice postnatal repeated mild stress (daily removal from the mother from birth to 21 days of age for 10 min plus subcutaneous injection of vehicle or drugs) induces an increase in body weight and an alteration in analgesic threshold (Loizzo et al. (2002) Br J Pharmacol 135: 1219–1226). Therefore, we evaluated whether taurine administration to offspring during weaning period (50 mg/kg per day) is able to prevent the stress-induced residual alterations in the adult life. In this experiment two parameters were considered.

First parameter. At 30 and at 40 days of age stressed mice showed an enhanced latency to response at nociceptive test (hot-plate) versus controls, as expected. In both occasions taurine treatment was able to prevent this effect, and to report peripheral pain response to normal values.

Second parameter. At 90 days of age, stressed mice showed a consistent decrease of immobility time in the forced swimming test versus controls, while taurine was not able to prevent this condition.

These data confirm and extend previous indications in the literature, on the antagonistic properties of taurine, versus selected alterations induced by early stress in the adult life, even when it is administered at very low doses. Further experiments are in progress to verify: 1) the possible mechanisms of these effects, and 2) the dose-effect relationship of taurine administration.

Oxidation of bacterial surfaces, a decisive step in microbicidal action

W. Gottardi and **M. Nagl**

Institute of Hygiene and Social Medicine, Leopold-Franzens-University of Innsbruck, Austria

If bacteria are incubated with solutions of active chlorine compounds, oxidation capacity in form of N–Cl bonds (“chlorine cover”) is fixed at the cell surface.

Experiments with the mild oxidant N-chlorotaurine show that bacteria can tolerate well measurable chlorine covers without a marked loss of viability.

At high-level chlorine covers, however, as is the case with chloramine T and chiefly sodium dichloroisocaynurate, very fast killing takes place which suggests a destructive impact on the cell wall if the bacterial surface is stud with too much N–Cl bonds.

According to the present understanding the operation of active chlorine compounds on bacteria can be divided into a non-lethal and a lethal section. The former implies reversible alterations at the bacterial surface and manifests itself by the development of a noticeable chlorine cover and a postantibiotic effect. The latter, however, is connected with irreversible alterations combined with a continuous increase of chlorine cover strength which finally leads to bacterial death.

Taurine chloramine triggers p53-dependent pathway leading to the cell-cycle arrest of rheumatoid arthritis synoviocytes

E. Kontny[1], **W. Rudnicka**[1], **M. Chorąży-Massalska**[1], **I. Janicka**[1], **J. Kowalczewski**[1], **J. Marcinkiewicz**[2], and **W. Maśliński**[1]

[1] Institute of Rheumatology, Warsaw, Poland
[2] Jagiellonian University, Cracow, Poland

Fibroblast-like synoviocytes (FLS) are active participants of chronic synovitis and synovial membrane hyperplasia, characteristics of rheumatoid arthritis (RA). We have previously reported that taurine chloramine (Tau-Cl) exerts both anti-inflammatory and anti-proliferative effects on RA FLS. In present study the mechanism of Tau-Cl inhibition of cell proliferation was investigated. FLS isolated from synovial membrane of RA patients were stimulated with either PDGF (10 ng/ml) alone or together with Tau-Cl (200–500 μM). Cell proliferation (^{3}HTdR incorporation and flow cytometric analysis of dividing cells stained with CFSE), the expression of proteins (Western blotting) responsible for cell-cycle arrest at either G1 (p21) or G1 and G2 (p27) checkpoints, as well as the expression of proliferating cell nuclear antigen (PCNA) indispensable for G1/S phase progression were evaluated after 24–72 h of cell treatment. Expression of p53 protein was examined in the cytosolic and nuclear fractions obtained after 2–24 h treatment of the cells with Tau-Cl alone. Tau-Cl inhibited both cell proliferation and PCNA expression ($IC_{50} \approx 250$–$300\ \mu$M), while up-regulated levels of mitotic inhibitors (p21 and p27). Importantly, at early time points (2–8 h) Tau-Cl significantly raised p53 expression in the nuclear fraction. We report that Tau-Cl induces an early cell-cycle arrest of RA FLS acting mostly via p53-dependent pathway, for this compound up-regulates p53 level and changes expression of proteins (PCNA, p21) known to be controlled by p53 transcription factor. We suggest that application of Tau-Cl could be considered as a potent agent that prevents an uncontrolled cell growth in pathological conditions, including RA.

Taurine concentration in the brain and in the plasma following taurine i.p. injection

Frédéric Lallemand and **Philippe De Witte**

Biologie du Comportement – Université catholique de Louvain, 1 Place Croix du Sud, 1348 Louvain-la-Neuve, Belgium

Taurine, a sulphonated β amino acid is ubiquitously present in mammals body, its concentration differs depending on the body part (i.e. high in the brain and heart, and low in the body fluids). Taurine is uptaked from the blood vessel to the cells by an active transport with a high affinity, Na^+ and Cl^- dependent transporter.

In our present study, the concentration of an ip injection of taurine at different doses (0.05, 0.125, 0.250, 0.5 and 1 g/kg) was investigated in both hippocampal microdialysates and retro-orbital sinus plasma samples.

The results showed a significant increase of taurine concentration both in the hippocampal microdialysate content (i.e. 1.97 mM and 13.5 mM after 0.125 g/kg and 1 g/kg ip injection from a basal concentration of 468 μM) and in the plasma (1.07 mM and 5.48 mM after respectively 0.125 g/kg and 1 g/kg ip injection from a basal concentration of 375 μM).

These increases were thus dose dependent and a greater amount of injected taurine appeared to be present into the brain rather that in the plasma.

Ethanol but not acetaldehyde induced changes in brain taurine: a microdialysis study

Vladimir A. Kashkin[1], **Frédéric Lallemand**[2], and **Philippe De Witte**[2]

[1] Valdman Institute of Pharmacology, Pavlov Medical University, St.-Petersburg, 197089, Russia
[2] Biologie du Comportement – Université catholique de Louvain, 1 Place Croix du Sud, 1348 Louvain-la-Neuve, Belgium

Research has suggested that catalase plays a role in mediating ethanol’s psychopharmacological effects. Catalase is an enzyme that oxidizes ethanol to acetaldehyde (ACH). It has been reported that when catalase activity is reduced by 3-amino-1,2,4-triazole (AT), rats reduce their intake and preference for ethanol. The present study assessed the effects of AT on the brain amino acids levels following ethanol administration in Wistar rats. The study consisted of three parts. In the first part, we found

no effects of acute and chronic intraperitoneally administered ACH on amino acids dialysate levels in nucleus accumbens. In the second part, AT was administered five hours prior to ethanol or its vehicle. Ethanol significantly affected the levels of taurine in rat pre-treated with AT. In the final part, ethanol was administered following the pre-treatment with AT but the dependent variable was the concentration of ethanol in the brain.

Taurine: evidence of a physiological function in the retina

J. B. Lombardini and **Julius Militante**

Department of Pharmacology, Texas Tech University Health Sciences Center, Lubbock, Texas, 79430, USA

Taurine is found in the highest concentration in the retina where levels as high as 80 mM have been reported. Taurine depletion results in retinal pathology usually observed in an attenuation of the electroretinogram signal and a disruption of the rod outer segments in the photoreceptor layer.

The high levels of taurine in the retina may be required for protection against oxidative stress. Taurine is known to inhibit light-induced lipid peroxidation and to form taurine chloramine which reduces the concentrations of hypochlorous acid.

Taurine has been demonstrated to stimulate ATP-dependent calcium uptake. The closure of cyclic GMP (cGMP)-gated cation channels, the net extrusion of calcium, and the decrease in intracellular calcium that occur during photoexcitation lead to the activation of a calcium-sensitive guanylyl cyclase and to an increase in cGMP. The activation of cGMP-gated channels allows for the increased entry of calcium. The increased calcium level results in the inhibition of guanylyl cyclase and in the breakdown of GMP by phosphodiesterase, allowing for recovery from the hyperpolarized state of the photoreceptor. Taurine may assist in this recovery by increasing calcium uptake through the cGMP-gated channels.

Our laboratory has also demonstrated that taurine inhibits the phosphorylation of a specific protein in the rat retina. Isolation of this specific protein suggests that it is histone H2B which would indicate that taurine has a regulatory role in DNA replication.

Attenuation of virulence of bacteria and fungi by N-chlorotaurine

M. Nagl, G. Pilz, R. Arnitz, W. Gottardi, and **R. Würzner**

Institute of Hygiene and Social Medicine, Leopold-Franzens-University of Innsbruck, Austria

N-chlorotaurine (NCT) is a long-lived oxidant produced by activated human granulocytes and monocytes during inflammation. Because of its broad-spectrum microbicidal activity it is conceived to be involved in destruction of pathogens *in vivo* and, besides that, as a new topical antimicrobial agent useful in medicine to treat infections.

At short, sublethal incubation times in NCT solution Staphylococcus aureus and Streptococcus pyogenes have been demonstrated to loose virulence when applied intraperitoneally to mice. While control mice challenged with mock treated bacteria died within 24 hours, those challenged with NCT-treated bacteria survived without developing septicemia. Streptococci and to a lesser extent staphylococci attenuated by NCT were phagocytosed at a higher rate by granulocytes than control bacteria, and their regrowth was delayed in broth solutions (postantibiotic effect).

A similar lag of regrowth could also be observed in Candida spp. Moreover, the production or secretion of aspartyl proteinases, which are virulence factors of yeasts, was inhibited already by low concentrations of NCT which did not inhibit fungal growth. The adherence of Candida to epithelial cells decreased rapidly, too.

These results indicate a rapid loss of virulence of pathogens as the first step of mechanism of action of NCT, probably caused by oxidation of the microbial surface. This is an important aspect for clinical application of the compound.

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Efficacy of N-chlorotaurine in acute otitis externa compared to Otosporin®

Andreas Neher[1], **Michaela Gstöttner**[1], and **Markus Nagl**[2]

[1] Department of Otorhinolaryngology, University Hospital of Innsbruck, Austria

[2] Institute of Hygiene and Social Medicine, Leopold-Franzens-University of Innsbruck, Austria

Objectives: Testing of new antiseptics which are well tolerated and effective against pathogens causing external otitis.

Study design: The endogenous antimicrobial agent N-chlorotaurine (NCT) was compared with Otosporin® (containing neomycin, polymyxin B, hydrocortisone) for topical treatment of acute external otitis in a randomised and rater-blinded phase 2 clinical study.

Methods: A cotton strip was applied into the external ear canal and subsequently soaked with 1 ml of 1% aqueous NCT solution or Otosporin® (single-blind because of the high viscosity of the latter one) once daily in 25 patients per group until the symptoms disappeared. Efficacy and tolerability were evaluated in a double-blind fashion by otoscopy using a six-scale inflammation score, and by audiogram subsequent to therapy.

Results: Both medications were well tolerated. The inflammation score improved more rapidly in the NCT group, and this difference became highly significant on days 4 to 7 ($P < 0.01$ each). Time needed for disappearance of inflammation (score zero) was 5.6 ± 1.6 days (mean $\pm$ SD, range 3–9 days) in the NCT group, and 7.4 ± 1.6 days (range 4–10 days) in the Otosporin® group ($P < 0.001$). As expected, microbiological cultures from ear swabs revealed *Pseudomonas aeruginosa* (58%) followed by *Staphylococcus aureus* (18%) as the main causative pathogens. All patients noted normal hearing after healing, which was confirmed by audiogram in 8 subjects (4 of each group).

Conclusions: NCT appears to be well tolerated and more effective than the standard therapy in acute external otitis. Further controlled studies should be performed to confirm these encouraging results in a larger population.

Modulation of taurine release by adenosine in the mouse hippocampus

Simo S. Oja and **Pirjo Saransaari**

Brain Research Center, University of Tampere Medical School, Finland

The neuromodulator adenosine is known to affect the release of neurotransmitters, e.g. that of glutamate. Adenosine is therefore thought to be an endogenous protective agent against cerebral ischemia and excitotoxicity. Of four adenosine receptors A_1, A_{2A}, A_{2B} and A_3, the first are particularly involved in the regulation of neurotransmitter release. Ischemic conditions markedly enhance the release of excitatatory amino acids together with adenosine release. Ischemia also induces in both

adult and developing brain the release of taurine, an inhibitory neuromodulator. We studied now the effects of agonists and antagonists of adenosine receptors and adenosine transport inhibitors on [^{3}H]taurine release from hippocampal slices. Under standard conditions, the adenosine A_1 receptor agonists N^6-cyclohexyladenosine and R(−)N^6-(2-phenylisopropyl)adenosine enhanced the basal release in developing mice and depressed the release in adults in a receptor-mediated manner. The compounds affecting adenosine A_2 receptors had only minor effects on basal release and did not affect K^+-stimulated release. In the immature hippocampus, the taurine release evoked by the ionotropic glutamate receptor agonist N-methyl-D-aspartate was enhanced by A_1 agonists in a receptor-mediated manner. In ischemia, the adenosine compounds had no marked effects on taurine release in immature mice, but A_1 receptor activation was still able to evoke release in adults by a receptor-mediated mechanism. Furthermore, the release induced by free radical production (hydrogen peroxide) was reduced by N^6-cyclohexyladenosine, which effect was abolished by the antagonist 8-cyclopentyl-1,3-dipropylxanthine but only in the immature hippocampus. The A_1 receptors modulate taurine release in the mature and immature hippocampus in both normoxia and cell-damaging conditions.

Taurine and peptide chloramine mediated formation of RFT

S. Olszowski, D. Kusior, E. Olszowska, P. Płonka, J. Zagajewski, T. Stelmaszyńska, and **A. Niedźwiedź**

Jagiellonian University, Collegium Medicum, Institute of Medical Biochemistry, Kopernika 7, 31-034, Kraków, Poland

Stimulated neutrophils (PMNs) are a source of the hypochlorite ($HOCl/OCl^-$) which in turn could react with amino groups of amino acids, peptides and proteins yielding of variety of mono- and dichloramines. Chloramines formed at α-amino groups are unstable and undergo the deamination and decarboxylation processes with concomitant aldehyde formation. Others, like β-alanine or taurine chloramines exist in water solution within hours/days. It is likely, that, at the inflammation site, hypochlorite reacts with $–NH_2$ groups of N-terminal amino acids of peptides and proteins which are present in the medium. In model systems the peptide derived chloramines formation could be easily monitored by UV spectroscopy (mono- at 250 nm and di- at 300 nm), however, the knowledge about their relative reactivity is poor.

The aim of this study was to examine whether mono- and dichloramines of dipeptides containing aromatic residues (Phe, Tyr and Trp) could act as oxidants. Their relative reactivity was monitored with: (1) chemiluminescence (CL) method in luminol/hydrogen peroxide system, (2) oxidation of dihydrorhodamine, (3) oxidation of reduced cytochrome c, (4) semiquinone radical formation from hydroquinone (VIS at 470 nm and EPR method). Moreover, using the same methods, we examined reactivity of $HOCl/OCl^-$ – modified peptides but deprived of chloramine chlorine by reduction with methionine. Stable taurine and β-alanine mono- and dichloramines were used as a reference compounds.

It was found that dichloramines of taurine, β-alanine and dipeptides (Ala-Phe, Phe-Ala, Ala-Tyr and Tyr-Ala) exhibited comparable CL, semiquinone production, rate of cyt.c and dihydrorodamine oxidation whereas those containing Trp residue (Ala-Trp and Trp-Ala) gave no effect. Moreover dipeptides containing Trp or Tyr moieties were able to reduce cyt.c(Fe^{+3}). It suggest that chlorination of Tyr-Ala and Ala-Tyr affects free amino groups and probably the phenol moiety. Thus, those peptides could act as the oxidant (chloramine group) and as a reducing agent (functional group at the benzene ring). It is interesting that higher yield and rate of cyt.c reduction was observed in the case of Ala-Tyr. The tyrosyl radical signal (EPR) was also observed for Ala-Tyr but not for Tyr-Ala.

Methionine mediated decomposition of dipeptide chloramines decreased CL yield for all examined peptides and abolished semiquione formation. However, dipeptides containing Phe or Tyr still were able to oxidize luminol. This suggests that beside chloramines, hypochlorite induce (in aerobic conditions) formation of unstable products of ring peroxidation. On contrary, decomposition of chloramines at peptides containing Trp and Tyr increased their ability for cyt.c(Fe^{3+}) reduction. The peptide Phe-Ala exhibited the higher CL of all examined peptides suggesting the highest content of the peroxidation product. Thus, ESI spectra of the HPLC fractions were analyzed. The preliminary analysis revealed that the formation of higher molecular products (692, 507 etc.) from Phe-Ala (237) was observed. Thus, it could be concluded that not only hypochlorite mediated Tyr peroxidation leads to the covalent cross-linking in proteins (dityrosine) but also Phe could be involved.

It could be expected that at a inflammation site those peptides and proteins which contain at the N-terminal Phe, Tyr exhibit increased oxidative ability and even after chloramines decomposition could produce free radical intermediates (semiquinone radical). Thus, such protein ends induce the action of known xenobiotics (e.g. hydroquinone.). Trp, however acts as a reducing agent. It seems to be that tyrosine plays the double role – is an oxidant but after chloramines removal acts as a reducing agent.

Characterization and regulation of the taurine transporter and taurine biosynthetic enzymes in 3T3-L1 adipocytes

Hideo Satsu, Masakazu Takasaki, and **Makoto Shimizu**

Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Japan

The intracellular level of taurine is maintained both by the taurine transporter (TAUT) and by endogenous synthesis from methionine and cysteine in several tissues. Recently, it was reported that the taurine biosynthetic enzymes are highly expressed in rat adipose tissues. Therefore, we investigated the characteristics and regulation of TAUT and the cysteine dioxygenase (CDO), one of the major taurine biosynthetic enzymes, in 3T3-L1 adipocytes. In differentiated 3T3-L1 adipocytes, the activity of TAUT was evaluated by means of an sodium- and chloride-dependent high-affinity transport system, which was specific to β-amino acids. The activity of TAUT was up-regulated under hypertonic conditions, the expression level of TAUT mRNA being also increased. These phenomena were also observed in other tissues such as kidney, small intestine, and hepatocyte, suggesting that TAUT expressed in 3T3-L1 adipocyte is similar to that previously characterized in several tissues. When 3T3-L1 preadipocytes were cultured in the medium containing insulin, dexamethasone (DEX), and 3-isobutyl-1-methyl-xanthine (MIX), the activity of TAUT was significantly increased. Further after the medium was changed to the one containing only insulin, the activity of TAUT was gradually decreased. Kinetic analysis shows that this change of TAUT activity was due to the change of maximal velocity of TAUT. On the other hand, the expression level of CDO mRNA was gradually increased when the cells were cultured in the medium containing insulin, DEX, and MIX for 45 hours. Further 2 days culture of cells in the medium containing only insulin extremely increased the expression level of CDO mRNA.

Protective effects of taurine on the TNF-α-induced damage of intestinal epithelial cell monolayers

M. Shimizu, T. Mochizuki, and **H. Satsu**

Department of Applied Biological Chemistry, The University of Tokyo, Japan

The taurine transporter (TAUT) in human intestinal epithelial Caco-2 cells was up-regulated by incubating the cells with TNF-α (FEBS Lett., 517, 92–96, 2002). This up-regulation occurred at a transcriptional level and was diminished by treating the cells with NFκB inhibitors. We have

also found that a NFκB binding site is present in the upstream region of TAUT gene. These results suggest that NFκB pathway is likely to be involved in this regulation. The increase in the TAUT activity in the intestinal epithelial cells exposed to the inflammatory cytokines such as TNF-α and the following increase in the intracellular taurine concentration could be a physiological and protective response of the intestinal epithelial cells. To obtain a more direct evidence for the protective function of taurine in the inflammatory process of intestinal epithelial cells, a new model system was constructed by using Caco-2 cell monolayers. Caco-2 cells were monolayer-cultured on the semipermeable membrane, and then a human THP-1 cells differentiated into macrophage-like cells were co-cultured in the basal chamber of the Caco-2 cell monolayers. By co-culturing these cells for 48 hrs, increased release of LDH (lactate dehydrogenase) from the Caco-2 cells was observed. The integrity of the Caco-2 monolayers were also decreased, suggesting that the Caco-2 cell monolayers were damaged by culturing with differentiated THP-1 cells. The conditioned medium of the THP-1 cells gave a similar results. Interestingly, the LDH-release induced by the conditioned medium was almost completely inhibited by adding anti-TNF-α antibody, indicating that TNF-α secreted from the THP-1 cells played a major role in the damage of the Caco-2 cells. Addition of high concentrations of taurine to the culture media protected the Caco-2 cells from the damage. This model system will be useful to study the inflammatory damage of cells in the intestinal epithelium and the protective functions of taurine in this process.

Taurine and cytoprotection

Florence Ledecque, Sarah Fico, Robert R. Crichton, P. Heuschling, and **Roberta J. Ward**

Unite de Bichimie, University of Louvain, Louvain-la-Neuve, Belgium, University of Luxembourg, Luxembourg

Taurine capacity to act as an antioxidant has been attributed to its role in the detoxification of hypochlorous acid by the formation of taurine chloramine in addition to its ability to alter the activities of cytoprotective enzymes. The ability of alveolar macrophages and an immortilised micoglia cell line to respond to an inflammatory stimuli, lipopolysaccharide and interferon-gamma, after taurine loading has been investigated. Prior to stimulation the taurine loaded cells showed enhanced nitrite content, (equivalent to inducible nitric oxide synthase), which increased significantly after stimulation, but at a significantly lower level than that of controls. The activity of myeloperoxidase in both macrophages and microglia was undetectable, indicating that taurine-induced changes were not via the formation of taurine chloramine.

The reduction of the inflammatory response after taurine supplementation may be associated with decreased translocation of NFκB to the nucleus to bind with the consensus sequence in the promoter region of the iNOS gene or to the reduction of other pro-inflammatory agents.

Transglutaminases

Factor XIIIA subunit-deficient mice developed severe uterine bleeding events and subsequent spontaneous miscarriages

Akitada Ichinose[1], **Mitsunori Yamakawa**[2], **Gerhard Dickneite**[3], and **Shiori Koseki-Kuno**[1]

[1] Department of Molecular Patho-Biochemistry and Patho-Biology, and [2] First Department of Pathology, Yamagata University School of Medicine, Yamagata, 990-9585 Japan

[3] Preclinical Pharmacology and Toxicology, Aventis Behring, Marburg, Germany

Factor XIII (XIII) deficiency is caused by defects in the gene for it's a subunit (XIIIA), a member of the transglutaminase family, or B subunit (XIIIB), and leads to a life-long bleeding tendency and spontaneous miscarriage in female patients. In order to understand the molecular pathology of the latter symptom *in vivo*, we functionally analyzed XIIIA knockout (KO) mice. Several types of breeding pairs were tested for fertility using XIIIA KO and wild-type mice. All XIIIA KO female mice were capable of becoming pregnant, but most of them died due to excessive vaginal bleeding during gestation. Abdominal incisions revealed that the uteri of the dead mice were filled with blood and that some embryos were much smaller than others within a single animal. Histopathological analyses of the small embryos revealed intrauterine fetal death (IUFD) in the dead XIIIA KO female mice. Furthermore, a series of histological examinations of the pregnant animals suggested that massive placental hemorrhage and subsequent necrosis developed in the uteri of the XIIIA KO mice on day 10.5 of gestation. This was true regardless of the genotypes of male mating partners and those of fetuses. These results are reminiscent of spontaneous miscarriage in human female patients with factor XIII deficiency, and indicate that the maternal XIII plays a critical role in uterine hemostasis and maintaining the placenta during gestation.

Inhibition of transglutaminase activity in kidney proximal tubular cells reduces extracellular matrix accumulation in response to high glucose levels typical of diabetic nephropathy

M. Griffin[1], **N. J. Skill**[1,2], **I. Coutts**[1,2], **M. Fisher**[2], **L. Huang**[2], **R. Jones**[1], **A. M. El Nahas**[2], and **T. S. Johnson**[2]

[1] School of Science, The Nottingham Trent University, Nottingham, UK

[2] Sheffield Kidney Institute, Northern General Hospital, Sheffield, UK

Kidney scarring associated with diabetic nephropathy (DN), is characterised by thickening of tubular and glomerular basement membranes of the kidney, suggesting enhanced synthesis and deposition of extracellular matrix (ECM) components.

As a cellular model to study tissue transglutaminase (tTG) involvement in diabetic nephropathy, we exposed OK proximal tubular cells to 36 mM glucose over a 96 h period. ECM deposited per cell doubled under these conditions compared to cells grown in 6 mM glucose. Increases in ECM deposition were not TGFβ_1 mediated but were mediated by increases in mRNA of collagen III, IV and fibronectin. This was accompanied by a mRNA dependent increase in tTG activity leading to a 25% increase in ECM associated tTG and a 150% increase in matrix associated $\varepsilon(\gamma$-glutamyl)lysine crosslink. Application of a site directed irreversible inhibitor of TG (2-[2-oxopropyl)thio]imidazolium derivative: R283) normalised tTG activity with a corresponding non mRNA dependent reduction in ECM deposition. This suggests a direct action of tTG mediated crosslinking on ECM accumulation and opens up possible therapeutic applications for the treatment of DN which will be discussed.

Transglutaminase activity in programmed cell death: the spermine role in preventing leaves of nutritional interest from degradation

A. Di Sandro, S. Casali, and **D. Serafini-Fracassini**

Dipartimento di Biologia e.s., Università degli Studi di Bologna, Bologna, Italia

Freshly-excised *Valeriana* leaves, which are commercially utilised for food purposes, have a short bench shelf. Therefore, in order to prevent their rapid degradation, spermine was exploited to treat entire plants, excised leaves and leaf-discs as this polyamine is known as the most effective anti-senescence agent. The spermine treatment was performed with concentrations included in the micro- and milli-molar range and for different exposure-times (up to 5 days). Spermine induced a protective effect only in the micromolar range, by achieving the highest protein and chlorophyll prevention from degradation at 200 μM. To further elucidate if the action of the spermine was mediated by the covalent binding of this polyamine to green tissue proteins, transglutaminase activity was evaluated by both colorimetric and radiometric assays. An endogenous calcium-dependent and DTT-activated transglutaminase activity was found higher in protein extracts of three day-treated discs in respect to zero time and five day-treated discs. Long term dialyses (4 days) enhanced the enzyme activity. The spermine-treated discs showed an higher amount of high molecular mass aggregates.

As the most of the chlorophyll is bound to the apoproteins of antenna complexes located in thylakoid membranes of chloroplasts, the main chloropyll binding proteins were partially isolated by sucrose gradients. Transglutaminase activity was detected both in photosystem-enriched fractions. The enzyme was partially co-purified with the main light-harvesting complexes (products of *lhc* genes): different SDS-PAGE bands, identified around 60–70 kDa, resulted immuno-reactive with two antibodies raised against transglutaminases of animal origin. These transglutaminase activities were calcium-dependent, DTT-activated and light stimulated.

Heparan sulfate proteoglycans are likely cell-surface receptors for matrix tissue transglutaminase

Elisabetta A. M. Verderio[1], **Dilek Telci**[1], **Lucy Hughes**[1], **Gerry Melino**[2], and **Martin Griffin**[1]

[1] School of Science, Nottingham Trent University, Clifton Lane, Nottingham, UK

[2] IDI-IRCCS, University of Rome ''Tor Vergata'', Rome, Italy

Cell-adhesion to the extracellular matrix (ECM) via assembly of focal adhesions and associated actin-stress fibers is one important regulator of cell survival. We previously demonstrated that matrices of fibronectin (FN) in complex with the FN binding protein tissue transglutaminase (tTG, Type II), prepared either *in vitro* or by cell-secretion forming a physiological ECM (tTG-FN), can largely restore loss of cell adhesion following inhibition of the classical FN ArgGlyAsp (RGD)-dependent adhesion pathway mediated by $\alpha_5\beta_1$ integrin receptors. RGD-independent adhesion to tTG-FN induces assembly of distinctive GTPase RhoA-dependent focal adhesions and associated actin-stress fibers, activates focal adhesion kinase (FAK) and critically depends on protein kinase Cα (PKCα), which is membrane-translocated in a RGD-independent manner. Further support for a role for tTG-FN in adhesion-mediated cell signalling was found by direct fluorescence of the actin-stress fibers, showing that a matrix of tTG-FN leads to a partial shift in the altered pattern of actin cytoskeleton found in tTG-deficient mouse embryonic fibroblasts towards the normal pattern of organised stress fibers visualised in wild-type fibroblasts. The outside-in signalling induced by tTG-FN results from the interaction of tTG with the cell-surface, since blocking of tTG accessibility by a monoclonal antibody greatly reduces this process. Moreover, it is specifically diminished by treatment of cells with heparitinase and not chondroitinaseABC, suggesting cell-surface heparan sulfate proteoglycans (HSPG) as the class of receptors which bind the tTG-FN matrix. The involvement of syndecan-4, the only HSPG that is a widespread component of focal adhesions and specifically signals through PKCα, is currently being investigated.

Characterization of the genetic diversity of microbial transglutaminase from *Streptomyces* species

M. J. Arrizubieta, E. Beaumont, K. Insausti, and **M. J. Beriain**

Departamento de Ciencias del Medio Natural. Universidad Publica de Navarra, Pamplona, Spain

The microbial transglutaminase from *Streptomyces mobaraensis* represents a unique type of transglutaminase that has appeared through an evolutionary pathway independent from the path that gave rise to transglutaminase enzymes in animals and other bacteria. The solved X-ray structure of this enzyme has shown that even its catalytic mechanism differs from previously characterized human and fish transglutaminases. In order to gain insight into this enzyme's structure-function, we have aimed at characterizing isozymes naturally present in other *Streptomyces* species. We observe that the MTG gene is widely distributed in the tested species, although enzymatic activity could be detected in the culture supernatant of only a small subset. We have cloned and sequenced MTG gene homologues from 6 different species. Despite the high overall sequence homology, there are regions that accumulate considerable number of differences between the characterized genes. The most variable region is the one coding for the signal peptide and the N-terminus of the mature protein. Interestingly, in the 3D-structure of the *S. mobaraense* MTG this N-terminal region corresponds to a loop positioned in close spatial proximity to the catalytic center of the enzyme. The possible evolutionary/functional significance of these differences is discussed.

A cytosolyc and a nuclear function of tissue transglutaminase

Z. Balajthy and **L. Fésüs**

University of Debrecen, Medical and Health Science Center, Faculty of Medicine, Department of Biochemistry and Molecular Biology, Debrecen, Hungary

Acute promielocytic leukemia (APL), characterized by differentiation arrest of granulopoesis at the promyelocytic stage, is the first human malignancy that can be efficiently treated with a cell differentiation inducer, all-trans retinoic acid (ATRA). Transglutaminases are a family of Ca^{2+}-dependent enzymes that mediate covalent cross-linking of proteins by forming amide bonds between glutamines and ε-amino groups of lysine residues. Although TG2 is essentially a cytosolyc protein, several lines of evidence suggest that the remarkable part of its cytosolyc pool are present in the nucleus.

Neutrophils and other phagocytic cells produce superoxide anions plus a range of other reactive oxygen species besides that they have to migrate to the target micro-organism.

Here we report that (a) TG2 translocates into the nucleus during neutrophil granulocyte differentiation process in NB4 cells; (b) inhibition of transamidation activity of TG2 during differentiation process by monodansylcadaverin results in, both in cytosol and nucleus, a degreased amount of protein cross-links which led to diminished neutrophil cellular functions, like NBT posivity and superoxide anion production; (c) at the same time the mRNA expression of $gp91^{phox}$ is diminished in the presence of TG2 inhibitor in maturing NB4 cells; (d) missing TG2 activity

in TG2$^{-/-}$ mice neutrophil cells lead to a significant decrease in gp91^{phox} protein expression, indicating an important role of this enzyme in the regulation of gene expression. Here we also report that the neutrophils, which are the fastes crawling cells while they are seeking their target micro-organism are being hampered in their movement both in the presence of the TG2 inhibitor and in TG2$^{-/-}$ mice.

Properties of a transglutaminase isolated from germinating *Vicia faba* cotyledons

L. J. Durose, M. Griffin, and **P. L. R. Bonner**

Department of Life Sciences, The Nottingham Trent University, Nottingham NG11 8NS, UK

Transglutaminases (E.C.2.3.2.13) have been detected in a variety of different plant species and tissues. The transglutaminase in plants have been reported to be both calcium dependent and independent enzymes capable of forming γ-glutamyl polyamine bonds and ε-(γ-glutamyl) lysine crosslinks. We report the purification of a transglutaminase from germinating *Vicia faba* cotyledons using anion exchange, hydrophobic interaction and immunoaffinity chromatography. Throughout the purification the enzymic activity was monitored using a protein crosslinking assay developed at NTU. The transglutaminase from *Vicia faba* cotyledons has an apparent Mr of 80,000 (as estimated by gel filtration and SDS-PAGE) is activated at 10 μM free calcium and is thiol dependent. At sub-optimal calcium concentrations the activity is strongly inhibited by GTP suggesting this enzyme could also by regulated by nucleotides. Many of these propertiesare similar to the properties of type II tissue transglutaminase isolated from mammalian tissues. The purified enzyme will provide some of the necessary tools to further study the activity in plant tissues and eventually help to establish a role for the enzyme within plant tissue.

Predicting protein structure: the test case of TGAses

Rita Casadio

Interdepartmental Center for Biotechnological Research (CIRB) and Department of Biology, University of Bologna, Via Irnerio, 42, 40126 Bologna, Italy

Building by homology and protein fold recognition methods, particularly those based on the recognition of distant homology are becoming increasingly effective at predicting protein tertiary structure from sequence, as seen in the various CASP prediction experiments. However, despite this success, some degree of human intervention is often necessary to perform successfully a good fold recognition method. In this talk I will be presenting a protocol that we developed for predicting protein structure when even distant homology search fails in providing a template for building the 3D structure of the target. As an example, I will be discussing the strategy that recently led us to predict structures of TGAses from different sources.

Characterisation of transglutaminase activity in the pollen of *Malus domestica* (cv Golden Delicious)

S. Del Duca[1], **A. A. Di Sandro**[1], **M. Griffin**[2], **D. Serafini-Fracassini**[1], and **P. L. R. Bonner**[2]

[1] Dipartimento di Biologia e.s., Università degli Studi di Bologna, Bologna, Italy

[2] Department of Life Science, The Nottingham Trent University, Nottingham, UK

The pollen grain has emerged as a model system in plant developmental studies thanks to two distinguishing features: its homogeneity and synchronicity of growth. During germination, the pollen grain develops a tube, which guides the male nucleus on its course to reach the female gamete. In previous work we identified cytoskeletal proteins (actin and tubulin) as transglutaminase substrates during pollen germination.

To characterise transglutaminase in extracts from pollen of *Malus domestica*, the activity of the enzyme was determined by a biotin cadaverine amine incorporation assay and a biotin casein protein crosslinking assay. The results showed the presence of a Ca^{2+}-dependent enzyme. Inhibitors of mammalian transglutaminase, cystamine, iodoacetamide, phenanthroline and active site directed inhibitor R283 decreased the pollen enzymes activity, whereas DIECA (an inhibitor of diamine oxidase) did not. Mono- and bis-(γ-glutamyl) putrescine derivatives were identified following incubation of pollen extracts in the presence of radioactive putrescine. Different amounts of the radioactive products were identified according to the physiological stage of germinating pollen.

Pollen transglutaminase has been partially purified using calcium dependent hydrophobic interaction chromatography and affinity chromatography using casein and spermidine. A 80 kDa protein was identified with a monoclonal antibody originally raised against guinea pig liver transglutaminase (ID-10).

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AtPng1p, a gene with a transglutaminase-like domain in plants. Cloning, purification and preliminary characterisation of its transglutaminase activity

M. Della Mea[1], **D. Caparrós Ruiz**[2], **I. Claparols Carrera**[2], **S. Fornalé**[1], **J. Rigau Lloveras**[2], and **D. Serafini-Fracassini**[1]

[1] Dipartimento di Biologia e.s., Università degli Studi di Bologna, Bologna, Italy

[2] Institut de Biologia Molecular, CID-CSIC, Barcelona, Spain

The research on transglutaminases in plants has been greatly delayed by the non-availability of a purified and sequenced enzyme. By contrast, the activity and some substrates are well characterised in different organs and organelles of many plants. Recently, bioinformatic analysis has shown the existence of an ORF putatively coding for a peptide N-glycanase in *Arabidopsis thaliana* (AtPng1p). Interestingly, AtPng1p also contains a putative transglutaminase-like domain. To verify whether this domain has transglutaminase activity, its presumably full-length cDNA was cloned in different expression vectors: the pET28(a)+ and the pET22(b)+ the latter modified in our laboratory with a His-tag tail at the 5′ terminus for affinity purification. The last plasmid, provided with a periplasmic signal, was used for exporting the recombinant protein in a soluble form into the periplasm. Unfortunately, a protein of about 80 kDa was over-expressed by both vectors, but in an insoluble-manner. Therefore, the purification was performed in denaturing conditions. After the purification by a Ni^{2+} column, specific for His-tag tail, the protein was re-folded, and assayed for its putative transglutaminase activity. Similarly to other transglutaminases described so far, this protein also shows a Ca^{2+}-dependent transglutaminase-like activity *in vitro*. RT-PCR analysis showed the presence of the AtPng1p mRNA in *Arabidopsis* plants. Therefore, although still preliminary, to our knowledge this is the first indication of an *Arabidopsis thaliana* cDNA coding for a putative transglutaminase enzyme. At the physiological role of this bi-functional enzyme (transglutaminase and peptide N-glycanase) is at present under study.

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Germination of *Malus domestica* pollen and its *in vivo* transglutaminase activity are affected by the same regulators

A. Di Sandro[1], **S. Del Duca**[1], **E. Verderio**[2], **A. Hargreaves**[2], **M. Griffin**[2], **D. Serafini-Fracassini**[1], and **P. L. R. Bonner**[2]

[1] Dipartimento di Biologia e.s., Università degli Studi di Bologna, Bologna, Italy
[2] Department of Life Science, The Nottingham Trent University, Nottingham, UK

By optical and laser scanning confocal microscopy, intracellular pollen transglutaminase was localised in germinating pollen tubes following fixation (4% fomaldehyde), cell-wall digestion (cellulase, pectinase), permeabilisation (triton-X100), and immunolocalisation using ID10 monoclonal antibody (raised against guinea pig liver transglutaminase) followed by secondary antibodies. Transglutaminase was identified in the proximal part of the tube in ring-like structures. Tubulin (positive control), a known pollen transglutaminase substrate, was localised as microtubules around nuclei and the tip. The antibody raised against ε-(γ-glutamyl)lysine (Covalab) identified the isodipeptide along all the length of tubes and in germination pores. The detection of the product of transglutaminase-mediated crosslinking suggests that transglutaminase may play a key role in the development of the pollen tube.

Transglutaminase activity was found in non-permeabilised pollen allowed to germinate within microplate-wells pre-coated with N,N′ dimethylcasein in the presence of biotin cadaverine. The ID10 antibody inhibited the activity of not-permeabilised pollen and its germination.

These results indicate the existence of an extracellular or cell-wall associated transglutaminase in addition to a previously identified intracellular enzyme. This is the first time that an extracellular transglutaminase has been detected in higher plants. The extracellular transglutaminase activity was higher in the germinated than in the ungerminated pollen. Transglutaminase activity was also found at the optimal pH (6.5) of germination in the medium isolated from pollen.

Various regulators of transglutaminase activity; cystamine, iodoacetamide, active site inhibitor R283, EDTA, also inhibited germination of tubes in a dose dependent manner and affected the pollen morphology, confirming a role for transglutaminase in pollen germination.

Oligomeric and polymeric aggregates formed by proteins containing expanded polyglutamine

S. Iuchi[1], **G. Hoffner**[2,*], **P. Verbeke**[2,*], **P. Djian**[2], and **H. Green**[1]

[1] Department of Cell Biology, Harvard Medical School, Boston, MA 02115, USA
[2] UPR 2228 CNRS, UFR Biomédicale, Université René-Descartes, Paris, France

Neurological diseases resulting from proteins containing expanded polyglutamine are characteristically associated with insoluble neuronal inclusions, usually intranuclear, and with neuronal death. We describe here oligomeric and polymeric aggregates formed in cells by expanded polyglutamine. These aggregates are not dissociated by concentrated formic acid, an extremely effective solvent for otherwise insoluble proteins. Perinuclear inclusions formed in cultured cells by expanded polyglutamine can be completely dissolved in concentrated formic acid, but a soluble protein oligomer containing the expanded polyglutamine and released by the formic acid is not dissociated to monomer. In Huntington Disease, a formic acid-resistant oligomer is present in cerebral cortex, but not in cerebellum. Cortical nuclei contain a polymeric aggregate of expanded polyglutamine that is insoluble in formic acid, does not enter polyacrylamide gels but is retained on filters. This shows that the process of polymerization is more advanced in the cerebral cortex than in cultured cells. The resistance of oligomer and polymer to formic acid suggests the participation of covalent bonds in their stabilization.

*These authors contributed equally.

Tissue transglutaminase modulators regulate actin rearrangements and cell cycle in several cell types

Caputo Ivana[1], **Barone Maria Vittoria**[1], **Troncone Riccardo**[1,2], **Auricchio Salvatore**[1,2], and **Esposito Carla**[3]

[1] European Laboratory for the Investigation of Food-Induced Diseases, University Federico II, Naples, Italy
[2] Department of Pediatrics, University Federico II, Naples, Italy
[3] Department of Chemistry, University of Salerno, Italy

Type II transglutaminase, also known as tissue transglutaminase, is an enzyme with multiple functions inside and outside cells. Among these, the enzyme seems to be involved in the receptor-mediated endocytosis of several ligands (Davies PJ et al., Mol Cell Biochem 1984, 58: 69–77). In particular, when agents that inhibit transglutaminase activity are used, they produce a biological interference with the endocytotic process. In the present study we aim to demonstrate that the inhibition of tissue transglutaminase activity can functionally interfere with some of growth factors-like mediated effects, such as cytoskeleton modification and regulation of cell cycle, in different cell lines. By adding the transglutaminase substrate monodansylcadaverine to the cell medium, we observe an increase of actin rearrangements in epithelial cells (Caco-2 and MCF7). As tissue transglutaminase is also distributed at the level of the extracellular cell membrane, we investigated the effect of modulating the activity of extracellular enzyme by incubating cells in the presence of the monoclonal blocking antibody clone CUB 7402. Interestingly, actin rearrangements similar to that evoked by monodansylcadaverine are obtained when the CUB 7402 is used. Moreover, both monodansylcadaverine and CUB 7402 are able to induce into S phase G_0 synchronised NIH 3T3 fibroblasts. In conclusion, our findings suggest a role for the outside membrane-associated tissue transglutaminase in the regulation of the growth factors trafficking in different cell lines.

Transglutaminase activity in free-living amoebae

D. Caccamo[2], **D. Forino**[1], **M. Currò**[2], **R. Ientile**[2], and **P. Ruggeri**[1]

[1] Departments of Environmental, Health, Social and Industrial Protection, Social Medicine and
[2] Biochemical, Physiological and Nutritional Sciences, University of Messina, Italy

Transglutaminases catalyze covalent cross-linking of proteins by forming isopeptide bonds between the γ-carboxamide group of glutamines and the primary amino group of lysines or polyamines. Transglutaminases are involved in many physiological functions, such as cell differentiation and senescence, apoptosis, wound healing, blood clotting, cell–cell signalling, and cell adhesion. Other than in vertebrates, transglutaminase homologues have been identified in parasitic nematodes and bacteria.

Free-living amoebae are unicellular eukaryotes, parasite of human and animal blood and tissues. Some species of *Acanthamoeba* and *Naegleria* genera are responsible for severe cerebral and ocular diseases, influenza-like syndromes and opportunistic infections in immunodeficient subjects. A crucial step in the ongoing of infections is the adhesion to host cells. Thus, the identification and inhibition of enzymes involved in cell adhesion mechanisms could offer new approaches for developing effective chemotherapeutic agents against these pathogens.

The aim of our work was to investigate, for the first time, the presence of transglutaminase activity in free-living amoebae, i.e. *Naegleria*

australiensis, *N. lovaniensis*, *Acanthamoeba castellanii*, *A. russ*, *A. polyphaga*. Enzyme activity was evaluated in the supernatant of cell homogenates by measuring the radioactivity of transglutaminase-mediated [^{3}H]-putrescine incorporation into cell proteins. The presence of transglutaminase was observed in all examined strains, which exhibited different levels of putrescine incorporation. Interestingly, the highest values of transglutaminase activity were observed in *N. australiensis* and *A. castellanii*, known for their capability of a stronger adhesion to host cells, in comparison to the other species. Effects of polyamines and transglutaminase inhibitors during *in vitro* culture were also evaluated.

Membrane associated transglutaminase in porcine brain

P. L. R. Bonner, R. Howden, M. Griffin, and **A. J. Hargreaves**

School of Science, The Nottingham Trent University, Clifton Lane, Nottingham, NG11 8NS, UK

There is increasing evidence to support the role of transglutaminase in development and disease in the mammalian nervous system. Moreover, it has recently been suggested that brain specific forms of tTGase may exist. The aim of this work was to reveal the major isoforms of TGase in sub cellular membrane fractions from brain. This was achieved both by measurement of TGase activity, biochemical markers of cell fractions and by probing Western blots with monoclonal antibodies that recognise TGase. Transglutaminase was enriched in a fraction containing relatively high levels of cytochrome c oxidase activity and very low levels of lactate dehydrogenase. Western blotting analysis indicated the presence of an approximately 80 kDa polypeptide that cross reacted with monoclonal antoibodies against TGase. Current work is aimed at further characterisation of the biochemical properties and function of this membrane associated form of TGase.

A novel function of tissue-type transglutaminase: protein-disulfide isomerase

Go Hasegawa and **Yuji Saito**

Department of Biological Sciences, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Yokohama, Japan

Tissue-type transglutaminase (tTG) is a multifunctional protein that is unique among the members of transglutaminases (TGases). The well-known function of tTG is catalysis of an acyl transfer reaction to form an ε-(γ-glutamyl)lysine isopeptide bond, which is common among TGase family. tTG can also act as a signal transducing G-protein, $G\alpha_h$. It is generally believed that tTG acts as TGase in the presence of calcium, otherwise it acts as $G\alpha_h$ in the presence of nucleotides. tTG has to localize itself into the plasma membrane when it acts as $G\alpha_h$. However tTG is mainly found in cytosol where the concentration of calcium is low, and that of nucleotides is high. Therefore physiological function of tTG in cytosol is still unknown.

To clarify this question, here we report a novel function of tTG. It has an activity of protein disulfide isomerase (PDI). We have shown that tTG converts RNase A molecule from completely reduced/denatured inactive state to the native active enzyme, probably by forming disulfide bridges in the molecule. This activity of tTG was inhibited by bacitracin, which is a frequently used inhibitor of conventional PDI activity. Glutathione of oxidized form (GSSG) especially enhanced PDI activity of tTG, but the reduced form (GSH) did not. PDI activity of tTG was not inhibited by GSH until its concentration was 200-fold higher than GSSG, therefore tTG can exhibit PDI activity in reducing environment like cytosol. These results would suggest that it is quite possible for cytosolic tTG to function as PDI.

Transglutaminases in cerebral ischemia

R. Ientile[1], **D. Caccamo**[1], **M. A. Catania**[2], **M. Currò**[1], **M. C. Marciano**[2], and **G. Calapai**[2]

[1] Department of Biochemical, Physiological and Nutritional Sciences, and [2] Department of Clinical and Experimental Medicine and Pharmacology, University of Messina, Italy

In a number of neurodegenerative diseases (i.e. AD, HD, PD, etc.) transglutaminase activity (TGase), which catalyzes the post-translational modification of proteins by transamidation of available glutamine residues, increases and probably contributes to the formation of protein aggregates in affected brain. A common feature in these pathologies is the altered levels of excitatory amino acids. More recently, we demonstrated that activation of glutamate receptors increased TGase activity and tissue transglutaminase expression in primary cultures of both cerebellar granule cells and differentiated astroglial cells. Consistent with a substantial evidence linking excessive glutamate neurotransmission to the development of neuronal death, following cerebral ischemia, in this study we characterized changes in TGase evoked by ischemic events produced in vulnerable regions of the brain. We demonstrated that in the hippocampus of gerbils that are subjected to temporary (3 min) bilateral carotid occlusion TGase activity increased and reached maximum levels at 24 h of post-ischemic periods. In the cerebral cortex, in which cell damage has been shown to be moderate after ischemia, TGase activity did not significantly change after 24 h recirculation. We also demonstrated by RT-PCR that multiple TGase enzymes, such as TGase1, Tgase2 and TGase3 are involved in normal neuronal structure and function. However, TGase2 was mostly induced in the hippocampus of gerbils. Together these results correlate well with western blot analysis performed with monoclonal antibody against TGase2. Thus it can be supposed that TGase increases may be considerably involved in reversible cerebral ischemia when, especially in the hippocampus, intracellular calcium levels are sharply increased.

Expression of tissue transglutaminase and heme oxygenase in primary astroglial cells during differentiation

A. Campisi[1], **G. Li Volti**[1], **M. Currò**[2], **F. Mazza**[1], **G. Cannavò**[2], **G. Raciti**[1], **R. Avola**[1], **S. Macaione**[2], **A. Vanella**[1], and **R. Ientile**[2]

[1] Departments of Biological Chemistry, Medical Chemistry and Molecular Biology, University of Catania, and [2] Biochemical, Physiological and Nutritional Sciences, University of Messina, Italy

Tissue tranglutaminase, in addition to being a transamidating enzyme, has an important role in cell signal transduction and apoptosis as well as heme oxygenase. Calcium-dependent transglutaminase reactions and heme oxygenase are also implicated in several neurodegenerative diseases. The aim of the present study was to evaluate the expression of tissue tranglutaminase and heme oxygenase in untreated and glutamate-treated primary astroglial cells during differentiation and how these two systems impact on caspase-3 mediated apoptosis. We exposed astroglial cells to glutamate in the presence or absence of cystamine, a competitive inhibitor of transglutaminase activity, and $SnCl_2$ and tin-mesoporphyrin, inducer and inhibitor respectively of heme oxygenase activity. We demonstrated, using confocal laser scanning microscopy and western blot, that expression and subcellular localization of both transglutaminase and heme oxygenase significantly changed during astroglial differentiation. In addition, prolonged exposure to glutamate (100 μM) of astrocytes significantly increased the expression of both transglutaminase and heme oxygenase-1 and surprisingly their translocation into nuclear compartment. Glutamate exposure also increased caspase-3 compared to

control cells. However, pretreatment with $SnCl_2$ for 18 h inhibited glutamate mediated apoptosis. The addition of tin-mesoporphyrin stimulated apoptosis following 24 h of glutamate exposure. Furthermore, the addition of cystamine significantly reduced caspase-3 activation. Indeed, changes in tissue tranglutaminase expression were observed in glutamate-exposed astrocytes following treatments with heme oxygenase inducer and inhibitor, suggesting a possible link between these two enzymes in the metabolic machinery leading to protein modifications observed in neurodegenerative diseases.

Contribution of helices and transglutaminase-catalyzed covalent bonds to gelatin networks

Sébastien Giraudier and **Véronique Larreta-Garde**

Equipe de Recherche sur les Relations Matrice Extracellulaire – Cellules, Université de Cergy Pontoise, BP 222, 95302 Pontoise, France

Many biological gels involve both physical and chemical bonds. The relative influence of these two different kinds of links towards the gel properties are explored in model systems consisting of gelatin gels. Thermally reversible physical bonds, occurring with a coil to triple helix transition of gelatin chains, are obtained by cooling the protein solution. Covalent bonds are enzymatically catalyzed by a bacterial transglutaminase from *Streptoverticillium sp*. obtained from Ajinomoto.

Different temperature protocols were applied to gelatin solutions, with and without the enzyme, to vary the history of the gel. Different gels were thus obtained: ''physical'', ''covalent'' and ''mixed'' gels. The contribution of each type of network was investigated with use of rheology, polarimetry and enzymatic degradation.

Both the time for gel formation and the viscoelastic properties of the gel are concentration dependent. Covalent bonds limit, but do not totally prevent the formation of helices nor stabilize them. The gels properties are depending on both the order of formation and the relative quantity of weak interactions and covalent bonds. At a molecular point of view, enzyme degradation of gels implying covalent bonds is slower than that of physical gels, indicating a difference in the supramolecular organization of the gels.

Our study shows that a network with both helices and covalent bonds, such as the extracellular matrix, could be protected from the enzymatic hydrolysis while keeping its structure and dynamics which is of main biological relevance. This work is a contribution to the understanding of complex gel formation.

Ubiquitination of tissue transglutaminase is modulated by interferon-α in human lung cancer cells

Carla Esposito[1], **Monica Marra**[2], **Gaia GIuberti**[2], **Anna Maria D'Alessandro**[2], **Lucia Lubrano Lavadera**[3], **Giovanni Martin**[3], **Raffaele Porta**[3], **Anna Cozzolino**[3], **Michele Caraglia**[2], and **Alberto Abbruzzese**[2]

[1] Dipartimento di Chimica, Universita' di Salerno, Via S. Allende 1-84081 Baronissi, Salerno, Italy

[2] Dipartimento di Biochimica e BioÆsica, II Universita' di Napoli, Via Costantinopoli, 16 80138 Naples, Italy

[3] Dipartimento di Scienze degli Alimenti, Facolta 'di Agraria, Universita' 'Federico II', Portici, Italy

Tissue transglutaminase (tTG) is an enzyme involved in the regulation of apoptosis through the inactivation of some cell components. We demonstrated that Interferon-α (IFNα) induces apoptosis in human epidermoid cancer cells.

Here we report the effects of IFNα on the apoptosis and tTGAse modulation in human lung H1355 cancer cells.

The addition of 2500 I.U./ml IFNa for 48 h induced apoptosis, and caused an ~4-fold increase in the activity and expression of tTGase, in H1355 cells. However, the increase in mRNA levels for tTG was just 1.6-fold. On the basis of these data, we investigated whether tTG levels may be regulated through regulation of its degradation via ubiquitination. It was found that 2500 I.U./ml IFNa induced a time-dependent decrease in tTG ubiquitination. On the other hand, addition of the proteasome inhibitor lactacystin led to accumulation of the ubiquitinated form of the enzyme and to a consequent increase in its expression. Treatment of the cells with the two agents combined antagonized the accumulation of the ubiquitinated isoforms of tTG induced by lactacystin and caused a potentiation of tTG expression. Moreover, the tTG inducer retinoic acid was also able to increase expression and ubiquitination of tTG in H1355 cells. The addition of monodansylcadaverine (a tTG inhibitor) to IFNa-treated H1355 cells completely antagonized growth inhibition and apoptosis induced by the cytokine. In conclusion, we demonstrate for the first time that tTG is ubiquitinated and degraded by a proteasome-dependent pathway. Moreover, IFNa can, at least in part, induce apoptosis through the modulation of this pathway.

Building a low resolution model of a transglutaminase domain of an hypothetical N-glycanase from Arabidopsis thaliana

G. Tasco, M. Della Mea, D. Serafini-Fracassini, and **R. Casadio**

Dipartimento di Biologia e.s., Università degli Studi di Bologna, Bologna, Italy

TrEMBL classifies AtPng1p (Q9FGY9) as an hypothetical protein similar to a N-glycanase peptide. This protein contains a transglutaminase-like domain which is included in the transglutaminase family by Pfam (Protein families database). At the present, 147 proteins which share a tranglutaminase/transglutaminase-like domain are included in Pfam. This family includes both animal and bacterial transglutaminases as well as other proteins of unknown function. Sequence conservation in this superfamily primarily involves three motifs that centre around conserved cysteine, histidine, and aspartate residues which form the catalytic triad in the structurally characterised transglutaminase, namely the human blood clotting factor XIIIa (PDB code 1F13, the template resolution 0.21 nm).

Also the purified recombinant protein obtained from AtPng1p gene showed a calcium-dependent transglutaminase activity (see Della Mea et al. present issue), similarly to most of the transglutaminases. The alignment was obtained by the Clustal W tool (Blosum matrix was utilised and the default values of the gap penalty were maintained); the catalytic triad was aligned by the multiple alignment provided by Pfam. Both the sequence and the structure were considered. The modelling was built up by Modeller v6.2 and validated by Procheck v3.5.

Plant transglutaminase and changes in cytoskeletal proteins as markers of cryopreservation of plant tissue

Susan M. Trigwell[1], **Paul T. Lynch**[1], **Martin Griffin**[2], **Alan J. Hargreaves**[2], and **Philip L. R. Bonner**[2]

[1] Division of Biological Sciences, University of Derby, Kedleston Road, Derby. DE22 1GB, UK

[2] School of Science, The Nottingham Trent University, Clifton Lane, Nottingham. NG11 8NS, UK

Transglutaminase enzyme catalyses the Ca^{2+}-dependent formation of inter- and intra-molecular $\varepsilon(\gamma$-glutamyl) lysine iso-dipeptide bonds, and the post-translational modification of proteins by incorporation of polyamines. Transglutaminase activity has been detected in both the membrane and soluble fractions of higher plants. The enzyme has been shown to utilise a number of substrates, including cytoskeletal proteins.

Successful cryopreservation of plant material is important for a variety of ecological and commercial reasons, but the molecular basis of its success or failure is poorly understood. A previous study by our group investigated the correlation between pre-freeze treatment, post-thaw recovery level and transglutaminase activity in *Helianthus tuberosus* L. (Jerusalem artichoke) suspension cultures. Significantly higher enzyme activity was associated with the 0.5 M sucrose pre-freeze treatment, which in turn resulted in best post-thaw recovery. This implies a protective role for the transglutaminase during cryopreservation.

Our current project (part of a multi-discipline European objective to determine molecular markers of successful cryopreservation) aims to extend the above study to measure transglutaminase activity and changes in cytoskeletal proteins in extracts and sections of organised plant tissue. Plant tissue sections such as *Allium sativum* L. (garlic) stem discs, *Olea europaea* L. (olive) shoot tips and somatic embryos are commonly used for cryopreservation-based germplasm conservation. Preliminary results indicate the potential usefulness of transglutaminase activity and changes in cytoskeletal proteins as markers for successful cryopreservation.

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Isopeptide cross-links in Alzheimer's neurofibrillary tangles

Zoltán Nemes[1], **Levente Deli**[2], **Bart Devreese**[4], **Lyuben Marekov**[5], **Peter M. Steinert**[5], **Jozef vanBeeumen**[4], and **László Fésüs**[3]

[1] Department of Psychiatry,
[2] Department of Pathology, and
[3] Department of Biochemistry and Molecular Biology, University of Debrecen Medical and Health Sciences Center, Debrecen, Hungary
[4] Depatrtment of Biochemistry and Protein Engineering, University of Gent, Gent, Belgium
[5]Labortatory of Skin Biology, National Institute of Arthroses, Musculoskeletal and Skin Diseases, NIH, Bethesda, Maryland, USA

The formation of insoluble intracellular proteinaceous deposits is a common feature of numerous etiologically different neurodegenerative diseases, like Alzheimer's, Pick's, Parkinson's diseases, prion diseases, ALS, and many others.

Neurofibrillary tangle formation from tau, ubiquitin and other cytoplasmic proteins hallmark Alzheimer's disease pathology. Biochemical analysis of neurofibrillary tangles has yet been hampered by the insolubility of these highly cross-linked, insoluble, macro-polymeric proteinaceous neural deposits.

Transglutaminase-catalyzed covalent cross-linking of cellular proteins via $N^{\varepsilon}(\gamma$-glutamyl)lysine isopeptide bonds was suggested to be a crucial step in the formation of intracellular neurofibrillary aggregates.

We searched for the sequence localization of $N^{\varepsilon}(\gamma$-glutamyl)lysine cross-links in neurofibrillary tangle- enriched fraction from autopsy specimen of cortical brain tissue from patients with Alzheimer's dementia. Sarcosyl-insoluble neurofibrillary material was isolated from prefrontal cortices of autopsied patients with clinically and histologically verified cases of Alzheimer's dementia, dephosphorylated and affinity purified for ubiquitin moieties by magnetic separation. Tryptic fragments were separated by C18 reverse phase HPLC and were assayed for peptide bound $N^{\varepsilon}(\gamma$-glutamyl)lysine cross. HPLC eluate fractions containing cross-links were further sequenced by ESI MS/MS. Sequences were searched against the Swissprot, BLAST EST and Unigene Human databases.

Our data show that tau or neurofilament proteins are not involved in transglutaminase-mediated cross-link formation, but these are covalently cross-linked via (poly)ubiquitinyl moieties. Our data propose a generic molecular mechanism for the formation of macro-polymeric proteinaceous deposits in neural cells, a common hallmark of various primary neurodegenerative diseases.

Tissue transglutaminase over-expression inhibits tumor progression *in vivo*

Francesco Facchiano[1], **Alessandro Lentini**[2], and **Simone Beninati**[2]

[1] Laboratorio di Patologia Vascolare, Istituto Dermopatico dell'Immacolata, IDI-IRCCS, via Monti di Creta 104, 00167 Roma, Italy
[2] Dipartimento di Biologia, Università Tor Vergata, Via della Ricerca Scientifica, 00133 Roma, Italy

The effect of *in vivo* over-expression of tissue transglutaminase (TGase) was investigated by means of a gene therapy approach. In a murine model of cancer, namely intravenous injection of B16 melanoma cells in C57/BL6 mice, we evaluated the overall survival, number and size of lung metastases at 30th day after injection of melanoma cells. In control mice ($n = 20$), a PSG5 empty plasmid vector was injected into tail vein at 1st and 7th day after tumor cells injection, whereas at the same time other mice ($n = 20$) were injected with the same amount of the plasmid carrying the cDNA coding for human TGase-2 (PSG5-TGase-2). In these mice, a significant increase of total TGase activity was detected, as well as an increased TGase-2 expression was observed by Western blot analysis. While control mice showed the expected rate of mortality (i.e. 50% of mice were dead at 20th day after tumor injection, 100% mortality at 33th day), PSG5-TGase-2 treated mice were significantly less sensitive to melanoma (i.e. 50% of mice were dead at 20th day after tumor injection, 20–30% survival of mice at 33th day after injection). Furthermore, in mice sacrificed on 33th post-injection day, a significantly reduced number of lung metastases was detected (42% reduction in PSG5-TGase treated mice vs control mice). These data indicate that an increased enzymatic activity of TGase-2 may represent a novel and potent therapeutic strategy against cancer progression.

Role of the FAD-dependent polyamine oxidase in the selective formation of N^1,N^8-*bis*(γ-glutamyl)spermidine cross-links

A. Lentini, S. Balestrero, and **S. Beninati**

Department of Biology, University of Rome "Tor Vergata", Via della Ricerca Scientifica, 00133 Roma, Italy

Protein-bound γ-glutamylpolyamines have highlighted a new pathway in polyamine metabolism. The key role played by these conjugates on the conformation and biological activity of a protein is well documented by recent reports. In this sense, the specificity of the protein modification is very high because only protein-bound glutaminyl residues are the acceptor site of the primary amine group of polyamines. This property makes this posttranslational modification of proteins an useful tool to examine the biological role of polyamines. The levels of spermidine cross-links found in human epidermal foreskin keratinocytes, comparable with those of $\varepsilon(\gamma$-glutamyl)lysine, ascribes a functional role to this protein modification. However, the influence of the diverse cross-links on the mechanism of epidermal envelope assembly remains to be elucitated. We have found that, the selective oxidation of N^1-(γ-glutamyl)spermidine and N-(γ-glutamyl)spermine by FAD-dependent polyamine oxidase (PAO), may be one of the cellular mechanism regulating the preferential formation of a sterically defined N^1,N^8-*bis*(γ-glutamyl)-spermidine cross-links. These findings suggest that PAO may be one of the regulatory key enzymes, which function is to reduce alterations in epidermal envelope precursor proteins assembly.

Functional studies on transglutaminase 5

E. Candi, A. Paradisi, B. Cadot, and **G. Melino**

University of Rome Tor Vergata, Department of Experimental Medicine, via Montpelier, 1, 00133 Rome, Italy

Transglutaminases (TGase) are Ca^{2+}-dependent enzymes able to catalyze transamidation of specific glutamine residues to form intermolecular isopeptide bonds. Nine distinct TGases have been described in mammals: types 1–7, band 4.2 and coagulation factor XIIIa.

A new member of the TGase family, TGase 5, previously known also as TGase X, has been recently identified in differentiating keratinocytes. TGase 5 is expressed in different isoforms during the early stages of keratinocyte differentiation. TGase 5 is very efficient in using specific epidermal substrates such as loricrin, involucrin and SPRs. By indirect immunofluorescence analysis the antibodies decorated the upper layers of normal human epidermis, with consistent staining in the spinous and granular layers. TGase 5 contributes, as a secondary effect, to the hyperkeratotic phenotype in ichthyosis (both vulgaris and lamellar) and in psoriasis.

TGase 5 mRNA is not restricted to epidermal tissue, but is widely expressed in a variety of adult and fetal tissues including the nervous system, thymus and several glands. By comparative analysis of amino-acid alignments and by an homology-derived three-dimensional model, we identified a TGase 5 putative GTP-binding pocket. So far, only two members of the TGase family have been described to be regulated by guanine nucleotides: TGase 2 and TGase 3. TGase 2 is also able to hydrolyze GTP, therefore resulting in a bifunctional enzyme with both protein cross-linking and GTP-hydrolyzing activities. We report that TGase 5 is regulated by guanine/adenine nucleotides. GTP and ATP inhibit TGase 5 cross-linking activity *in vitro*, in a dose-dependent manner, and Ca^{2+} is able to completely reverse this inhibition. Taken together, these data indicate that TGases are a complex family of enzymes and in addition to calcium and nitric oxide, at least three of the nine known members are regulated by adenine and guanine nucleotides.

Role of transglutaminase 2 in glucose tolerance: knockout mice studies and a putative mutation in a MODY patient

Francesca Bernassola[1], **Fabrizio Barbetti**[2,3], **Massimo Federici**[4], and **Gerry Melino**[1]

[1] Biochemistry Laboratory, IDI-IRCCS, c/o Department of Experimental Medicine and Biochemical Sciences,
[2] IBCIT-Biomedical Science Park, 00144,
[3] IRCCS "Bambino Gesù" Children's Research Hospital, 00165, and
[4] Department of Internal Medicine, University of Rome "Tor Vergata", 00133, Rome, Italy

Transglutaminases are Ca^{2+}-dependent enzymes, catalyzing intermolecular isopeptide bonds between the gamma-carboxamide groups of peptide-bound glutamine residues and the primary amino groups of several compounds. The establishment of these covalent cross links leads to the post-translational modification of proteins by forming either $\varepsilon(\gamma$-glutamyl)lysine isodipeptides or N,N-bis(γ-glutamyl)polyamine linkages, and in many instances, the oligomerization of substrate proteins. At least seven distinct types of TGases have been characterized in mammals: TGase 1, TGase 2 (or tissue-TGase, tTG), TGase 3, TGase 4 (prostate), TGase 5, coagulation factor XIII, and band 4.2.

TGase 2 (EC 2.3.2.13) is a monomeric, cytosolic and membrane-associated protein, finely regulated at post-translational level, by Ca^{2+}, GTP and nitric oxide. In addition to its cross-linking activity, TGase 2 exhibits a signaling function as the Galphah subunit of a GTP-binding protein, modulating alpha1-adrenergic receptor-stimulated phospholipase C activation. The TGase 2 gene is constitutively expressed both during development and in adult tissues, where a tight correlation between TGase 2 expression and occurrence of apoptosis has been found. We have generated TGase 2-deficient mice through homologous recombination techniques. The mice are viable and fertile and show no developmental abnormalities. Apoptosis induced *in vitro* or *ex vivo* with different agents in fibroblasts and thymocytes respectively, appears to be normal.

The endocrine release of insulin occurs by exocytotic fusion with the plasma membrane and is triggered by a rise in intracellular calcium. The TGase 2 enzyme was previously implicated in a wide variety of both intracellular and extracellular biological processes, including stimulus-secretion coupling and receptor-mediated endocytosis of several proteins and hormones. A functional role for TGase 2 in Ca^{2+}-dependent glucose-stimulated insulin release from pancreatic beta-cells has been reported. TGase 2 also mediates retinoid-induced insulin secretion in rat islets. In addition, TGase 2 has been implicated in insulin-receptor aggregation, internalization, and intracellular processing, by cross-linking receptors in the area of clathrin-coated pits, thus participating in the regulation of insulin action in target tissues. Here, we show that targeted disruption of TGase 2 impairs glucose-stimulated insulin secretion. *TGase* $2^{-/-}$ mice show glucose intolerance after intraperitoneal glucose loading. In addition, *TGase* $2^{-/-}$ mice manifest a tendency to develop hypoglycemia after administration of exogenous insulin, as a consequence of enhanced insulin-receptor substrate-2 (IRS-2) phosphorylation. The increased peripheral sensitivity to insulin partially compensates for the defective secretion. *TGase* $2^{-/-}$ mouse phenotype is similar to the clinical features of the maturity-onset diabetes of the young (MODY) patients. Here, we report missense mutations in the active site of TGase 2 in a MODY-like patient. Collectively, these results identify TGase 2 as a candidate gene in Type 2 diabetes.

Production of recombinant transglutaminase in cereals

Teresa Capell[1], **M. A. Inmaculada Claparols**[2], **Stefano Del Duca**[3], **Ludovic Bassie**[1], **Berta Miro**[1], **Julian Rodriguez-Montesinos**[1], **Paul Christou**[1], and **Donatella Serafini-Fracassini**[3]

[1] Crop Genetics and Biotechnology Department, Fraunhomer IME, Auf dem Aberg 1, D-57392 Schmallenberg, Germany
[2] Molecular Genetics Department, Instituto de Biologia Molecular de Barcelona (IBMB)-Consejo Superior de Investigaciones Cientificas (C.S.I.C.), Jordi Girona 18-26, 08034 Barcelona, Spain
[3] Department of Biologia E.S., Università di Bologna, via Irnerio 42, 40126 Bologna, Italy

Higher plants are attractive hosts for the production of recombinant macromolecules for pharmaceutical, animal feed and nutraceutical applications. Plant-based production systems are inherently safer than alternative expression platforms and significantly more economical. An important additional advantage of plants is that they can carry out appropriate post-translational modifications of recombinant proteins. Cereal crops offer several advantages as expression systems. Recombinant proteins can be stored safely in protein bodies of endosperm cells, thereby escaping proteolysis in the cytosol, and subsequent programmed cell death during the final stages of cereal grain maturation. A wide diversity of high value recombinant proteins, with diagnostic or therapeutic potential, has been expressed in plants. We have recently expressed a recombinant transglutaminase in rice plants. The scarce source and the complicated separation and purification procedure for obtaining tissue transglutaminase result in an extremely high price of the enzyme. It is thus not feasible to utilize transglutaminase produced through conventional means in food processing on an industrial scale. In our current experiments, we wished to investigate whether transgenic rice tissues could accumulate a functional mammalian transglutaminase

in vegetative tissues and seeds. We introduced a mammalian transglutaminase ORF into rice. Transgene expression at the mRNA level was confirmed in leaf tissue by RT-PCR analysis. Western blot analysis demonstrated accumulation of the protein in leaves. Transgenic rice plants which accumulated the protein had a significant increase in transglutaminase activity. We thus established that rice plants can be a viable alternative to mammalian and prokaryotic expression systems for transglutaminase production.

Prolonged survival and reversal of abnormal movements in Huntington's Disease in a transgenic mouse model, after administration of cystamine, a transglutaminase inhibitor

Marcela V. Karpuj[1], **Mark W. Becher**[2], **Joe E. Springer**[3], and **Lawrence Steinman**[4]

[1] Institute of Neurodegenerative Diseases, UCSF, San Francisco, California, USA
[2] Department of Pathology, University of New Mexico Health Sciences Center, Albuquerque, New Mexico, USA
[3] Department of Anatomy and Neurobiology, University of Kentucky Medical Center, Lexington, Kentucky, USA

An expanded polyglutamine domain in huntingtin [htt] underlies the pathogenic events in Huntington's Disease [HD]. Transglutaminase [TGase] may be critical in the pathogenesis, via cross-linking and ''spot welding'' of htt. Administration of cystamine, a TGase inhibitor and competitor, to transgenic mice expressing exon 1 of htt, containing an expanded polyglutamine repeat, altered the course of their HD-like disease. Cystamine given intraperitoneally enters brain where it inhibits TGase activity. Beginning treatment after the appearance of abnormal movements, cystamine extended survival, reduced associated tremor and abnormal movements, and ameliorated weight loss. Treatment did not influence the appearance or frequency of neuronal nuclear inclusions, when started after the onset of abnormal movements. Others have shown that inclusions diminish when cystamine therapy is initiated earlier. Cystamine treatment increases transcription of one of the two genes shown to be neuroprotective for polyglutamine toxicity in Drosophila, dnaj [HDJ-1/Hsp40]. Inhibition of TGase provides a new treatment strategy for HD and other polyglutamine diseases.

''Tissue'' transglutaminase interactors in tissue injury

Pier G. Mastroberardino, Maria Grazia Farrace, Carlo Rodolfo, Serafina Oliverio, Irene Viti, Lucia Piredda, Federica Di Sano, and **Mauro Piacentini**

University of Rome ''Tor Vergata''-Via della Ricerca Scientifica, 00133, Rome, Italy

''Tissue'' transglutaminase or type 2 (TG2) is a multifunctional protein. The enzyme catalyses Ca^{2+}-dependent reactions in which the gamma-carboxamide groups of peptide-bound glutamine residues serve as acyl donors and primary amino groups of several compounds function as acceptor substrates. In its protein cross-linking configuration, TG2 plays a complex role in apoptosis, leading to the formation of cross-linked protein polymers which contributes to stabilising the integrity of dying cells before their clearance by phagocytosis. TG2 catalytic activity is enhanced by calcium ions and specifically inhibited by guanine nucleotides (GTP) and nitric oxide. Tissue transglutaminase is selectively activated also during development and under several pathological conditions. As a consequence of various cellular stimuli, different proteins act as TG2 interactors and substrates, which, as we previously showed, are located also on important cellular compartments such as mitochondria. TG2 catalysed crosslinking of different proteins under diverse pathological situations might cause inactivation of important prosurvival factors in the cell. Using a proteomic approach, we have identified various TG2 partners among which several chaperones, thus suggesting an important role for TG2 in post-translational folding of new-born peptides.

Addendum

From gene discovery to cancer treatment

C. M. Croce

Kimmel Cancer Ctr, BLSB, T. Jefferson University, Philadelphia, PA, USA

Fhit expression is reduced in most cancers and Fhit replacement by FHIT expression viruses in some lung, esophageal, pancreatic and cervical cancers causes apoptosis of the cancer cells. Mice carrying one or two inactivated Fhit alleles are hypersensitive to development of nitrosomethylbenzylamine (NMBA)-induced forestomach tumors. In a pilot study, tumor incidence was significantly reduced by oral delivery of FHIT expression viruses. In the present study, we investigated the kinetics and mechanism of tumor development, prevention and reversal. $Fhit^{+/-}$ mice received 6 intragastric doses of NMBA and then received no virus, GFP virus, or FHIT expression virus at 2, 21 and 42 days post NMBA. Analyses at various time points between 3 and 84 days post NMBA induced tumor incidence, multiplicity, size, histological and immunohistochemical assessment of expression of transduced Fhit and proliferation and apoptosis markers. Tumor analysis showed that: 1) by 37 days post NMBA control mice showed -7 tumors and by 84 days $\sim$10 tumors/forestomach; 2) mice receiving FHIT virus at 2 or 42 days post NMBA showed significantly reduced tumor burdens; 3) Fhit was still expressed at 82 post infection; 4) control viral infection had no effect on tumor development; 5) reduced Bcl2, increased Bax expression and increased TUNEL positive apoptotic nuclei characterization the restored epithelia of FHIT transduced forestomachs. Thus, FHIT viral gene therapy prevents or retards development of carcinogen induced forestomach tumors and reverse development of established tumors by 60–70% through an apoptotic pathway. This dramatic reduction in tumor burden emphasizes the efficacy of targeting the FHIT apoptotic pathway for tumor eradication.

Is nitric oxide involvement in the antinociceptive effects of Tyr-MIF-1 family of peptides in rats?

A. Bocheva, E. Dzambazova-Maximova, and **V. Petkov**

Institute of Physiology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria

The members of the Tyr-MIF-1 family include MIF-1, Tyr-MIF-1, Tyr-W-MIF-1 and Tyr-K-MIF-1. Tyr-MIF-1 and Tyr-W-MIF-1 have been isolated from bovine hypothalamus and parietal cortex of human tissue. Tyr-MIF-1 and Tyr-W-MIF-1 are able to interact with opioid receptors. Tyr-K-MIF-1 has been isolated from human brain cortex and appears to bind to Tyr-MIF-1 specific sites and to its own sites.

Nitric oxide (NO) appears to play a role a variety of biological events in the central nervous systems. Recent research has highlighted the role of NO in central nociception. Previous studies showed that L-NAME when administrated i.c.v. or systemically resulted in antinociception acting through the NO-cyclic GMP pathway.

The antinociceptive effects of the Tyr-MIF-1, Tyr-W-MIF-1 and Tyr-K-MIF-1 were studied during acute pain (paw-pressure test) in rats. Tyr-

MIF-1 (1 mg/kg, i.p.), Tyr-W-MIF-1 (1 mg/kg, i.p.) and Tyr-K-MIF-1 (1 mg/kg, i.p.) exerted the antinoinociceptive effects. The co-administration of Tyr-MIF-1 + L-NAME and Tyr-W-MIF-1 + L-NAME enhanced the nociceptive effects, which were decreased by SIN-1, naloxone and methylene blue. The results suggest the involvement of NO in the antinociceptive action of Tyr-MIF-1 and Tyr-W-MIF-1. The co-administration of Tyr-K-MIF-1 + L-NAME decreased the antinociceptive effect of Tyr-K-MIF-1.

The results suggest that NO might modulate the antinociceptive action of Tyr-MIF-1 family of peptides.

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Glutamine and tryptophan in relation to immunodepression during intensive training at altitude

Linda M. Castell[1], **Wayne Ensign**[2], **Jan Knight**[3], **Don Roberts**[2], **Doug Thake**[4], and **Dorothy Hudig**[5]

[1] University of Oxford, UK
[2] Naval Health Research Center, San Diego, USA
[3] Knight Scientific Ltd, Plymouth, UK
[4] School of Sports Science, Coventry, UK
[5] University of Nevada, Reno, USA

Introduction: Glutamine is important both as a fuel for some key immune cells, and in the metabolism of the antioxidant, glutathione. The incidence of upper respiratory tract infections (URTI) has been reduced in marathon runners and triathletes taking glutamine (or a glutamine precursor) vs. placebo after or during prolonged, exhaustive exercise. There is also a link between tryptophan availability and the immune response. The present study investigated plasma glutamine (p[Gln]), plasma free tryptophan (p[FT]), antioxidant capacity, some aspects of the immune system, and the incidence of illness in military personnel training at altitude up to 10,800 ft for 4 weeks (FTX). In a separate study p[Gln] was measured in healthy volunteers at rest and exercise, with and without hypoxia.

Methods: Sixty subjects consented to participate in the study. Blood samples were taken: 1) At baseline in the desert; 2) 1 day after arrival at 6,800 ft (FTX1); 3) Midway through FTX (FTX1b); 4) Immediately after FTX (FTX2); 5 & 6) 37 (Dsrt1) and 98 (Dsrt2) days after return to the desert. Samples were analysed for cell counts, cytokines, amino acids, and antioxidant capacity. The incidence of illness was monitored. In other studies, blood samples were taken from healthy controls at rest, before and after exercise (90 min, 50% VO_{2max}), with and without hypoxia, during a period of 5 hours.

Results: Compared with baseline samples: p[Gln] was decreased by 14% ($p < 0.001$) at FTX2, and by 7% at Dsrt1 ($p < 0.05$). Upper respiratory tract infections (URTI) increased substantially in most individuals at FTX2. p[Gln] was more markedly decreased at FTX2 (21%) in those who had the highest URTI scores. There was a marginally significant 9% lower antioxidant capacity at FTX1b *cf.* FTX1 and 2 ($p < 0.1$) and a 14% higher level at Dsrt1 ($p < 0.001$). In the studies with and without hypoxia at rest and in exercise, a slight increase in p[Gln] was seen after 2 hrs at rest but no other changes were observed.

Discussion: Decreases in p[Gln] and a relatively lower antioxidant capacity, occurred concomitantly with a high incidence of URTI. The decrease observed in p[Gln] at FTX2, together with the correlation between increased magnitude of the decrease and a higher URTI score, confirm previous data. In hypoxic conditions at rest and during exercise no decrease was observed in p[Gln]: thus it appears that the stress of prolonged, strenuous exercise may be more important than that of altitude. The decrease in p[Gln] at FTX2 might reflect a link between glutamine and/or glutathione availability and immunodepression.

Inhibitors of plant copper/TPQ amine oxidases

Jens Z. Pedersen[1], **Silvia Longu**[2], **Rosaria Medda**[2], **Alessandra Padiglia**[2], and **Giovanni Floris**[2]

[1] Department of Biology, University of Rome "Tor Vergata", Rome, and [2] Department of Applied Sciences in Biosystems, University of Cagliari, Cagliari, Italy

It is now well established that the active site of copper/TPQ amine oxidases contains Cu(II) and 6-hydroxydopa quinone (TPQ) as a prosthetic group, but the details of the catalytic process are still unknown. In particular little is known about the role of amino acid residues close to the active site, whether they participate in the reaction mechanism and how they determine the substrate specificity. Plant copper/TPQ amine oxidases often show a much higher catalytic activity than mammalian enzymes and are able to oxidise a broad range of amine compounds. Several synthetic and natural amines have been found to be both substrates and inhibitors of plant amine oxidases; some of these, like the haloethylamines, are toxic contaminants whereas others, like indoleamines such as tryptamine, are essential plant metabolites. We have studied the oxidative deamination of tyramine which is involved in the biosynthesis of some alkaloids and in the formation of hydroxycinnamoyl amines. These amines are widely distributed metabolites and are important for the plant defence against pathogens. Lentil seedling amine oxidase catalyzes the oxidation of tyramine, but the enzyme is irreversibly inactivated in the process and actually changes its usual pink colour to dark blue. The reaction mechanism by which tyramine inhibits lentil amine oxidase involves attack of the phenylacetaldehyde product on the ε-amino group of lysines to form a Schiff base adduct followed by the formation of several covalent derivatives. The results indicate that inactivation is due to modification of Lys296, suggesting that this lysine is important for the access of substrates to the active site.

Agmatine is transported in liver mitochondria by an energy-dependent mechanism

Antonio Toninello[1], **Mauro Salvi**[1], **Mario Mancon**[1], **Sebastiano Colombatto**[2], and **Maria Angelica Grillo**[2]

[1] Dipartimento di Chimica Biologica, Università di Padova, Istituto di Neuroscienze del C.N.R., Unità per lo Studio delle Biomembrane, Padova, Italy
[2] Dipartimento di Medicina e Oncologia Sperimentale, Università di Torino, Torino, Italy

Agmatine is a biogenic amine formed in "situ" by the decarboxylation of arginine in a reaction catalyzed by the specific enzyme arginine decarboxylase which is located in mitochondria.

Agmatine can also be taken up from exogenous sources, in fact it is present in food and is produced by the intestinal flora. The uptake has been demonstrated to take place in several different cell types. Its catabolism occurs, at least partially, by means of agmatinase, which produces urea and putrescine, and is also present in mitochondria. Mitochondria also contain the I_2 imidazoline receptor, whose function in the organelles is unknown at present, but is able to bind agmatine. Furthermore, recently it has also been reported that agmatine is present in mitochondria. All these observations led us to investigate whether agmatine can be transported in mitochondria and in this case to characterize the mechanism of uptake.

The obtained results demonstrate that agmatine is transported in rat liver mitochondria by an energy dependent mechanism. This mechanism is strictly $\Delta\Psi$ dependent being enhanced by the presence of phosphate or nigericin which with different mechanisms induce an increase of the electrical gradient, and is completely abolished by K^+ plus valinomycin

which induce a $\Delta\Psi$ collapse. $\Delta\Psi$ also influences the initial rate of agmatine transport showing an apparent exponential behaviour of the force-flux relationship.

The observation that agmatine, which is a divalent cation, is transported with a higher initial rate and extent than the tetravalent cation spermine, supports a possible involvement of the imidazoline receptor in the mechanism of agmatine transport.

Synthesis of novel 3-pyridinecarbonitriles with amino acid function and their fluorescence properties

A. Kalmouch and **A. S.Girgis**

National Research Centre, Cairo, Egypt

3-Pyridinecarbonitrile derivatives represent an important class of heterocyclic compounds characterized by versatile biological properties. Synthesis of novel 3-pyridinecarbonitrile with amino acid and/or peptide function as a natural product residue was investigated. Thus, *N*-[(4,6-diaryl-3-pyridinecarbonitrile)-2-yl] amino acid esters were synthesized through the reaction of 2-bromo-3-pyridinecarbonitriles with the appropriate α-primary (glycine, alanine) or secondary (sarcosine) amino acid ester hydrochlorides in refluxing dioxane in the presence of triethylamine as dehydrohalogenating agent. Similarly, *N*′-glycylglycine analogues were obtained through the reaction of the bromopyridines with the dipeptide derivative. On the other hand, reaction of the 2-bromopyridines with α-amino acids in refluxing pyridine gave the unexpected 2-amino-3-pyridinecarbonitriles. The fluorescence properties of the newly prepared pyridines were evaluated. Some of the synthesized pyridines show considerable antibacterial activity.

Mercury chloride inhibits creatine synthesis in the kidney

Jelenka Nikolic and **Dusan Sokolovic**

Institute of Biochemistry, Faculty of Medicine, University of Nis, Serbia and Montenegro

l-Arginine glycine transamidinase catalyses the production of glicociamine, which is the first, rate-limiting step in creatine synthesis. Enzyme is find mainly in the kidney and pancreas. Exposure to high doses of mercury chloride leads to symptoms of acute renal failure with dramatic symptoms of azotemia. In the rats treated with mercury chloride (3 mg/kg) the levels of urea and creatinine were increased 24 hours after administration of the $HgCl_2$, compared to control group ($p < 0.001$). Transamidinase activity in kidney tissue of intoxicated rats was significantly reduced ($p < 0.001$). Low level of transamidinase may be due to high levels of blood and kidney creatine which is released by increased tissue destruction as a result of adrenal steroids and catabolic stress on muscle mass. Depression of enzyme may occur as a result of the inhibitory effect of mercury chloride on enzymes molecules.

Synthesis of modified at position 1 at 3 analogues of endogenous tetrapeptide Tyr-MIF-1

Svetlana S. Pancheva, Rositca J. Kalauzka, Efrossina P. Popgeorgieva, and **Tamara I. Pajpanova**

Institute of Molecular Biology, Bulgarian Academy of Sciences, G. Bonchev Str. 21, Sofia 1113, Bulgaria

The N-terminal Tyr^1 ring is typical for endogenous opiate peptides and it is considered to be responsible for the opiate action. To understand more about correlation between modification of Tyr-sceleton and the biological activities of these tyrosine-like compounds, several peptide analogues based on the structure of the naturally occurring Tyr-MIF-1 (Tyr-Pro-Leu-Gly-NH_2) have been synthesized.

In this study we present five new analogues in which the $L\text{-}Tyr^1$-residue has been replaced with D-Tyr(Me). Following our previous work in design of Tyr-MIF-1 analogues, the Leu^3-residue has been substituted again with unnatural (sLeu, sIle, sNle) as well as nonproteinogenic (Cav, Can) amino acids. The synthesis is carried out in solution according to the general procedure of the peptide synthesis by the stepwise – chain building method.

The obtained peptides are subjected of pharmacological investigation giving evaluation their possibility of binding affinity and μ-selectivity.

Antimicrobial activities of amino acids and peptide-functionalized cholic acid

Ekaterina Slavcheva Grueva[1], **Tsenka S. Milkova**[2], and **Tamara I. Pajpanova**[1]

[1] Institute of Molecular Biology, Bulgarian Academy of Sciences, 1113-Sofia, 21, Acad. G. Bonchev, Str., Bulgaria

[2] Department of Chemistry South-West University ''Neofit Rilski'', 2700 Blagoevgrad, Bulgaria

Combinatorial chemistry, in conjunction with solid phase synthesis, is proving a powerful tool for the discovery of biological active molecules, receptors and catalysts.

As noted by several groups, the steroid nucleus has special attractions as a starting point for scaffold design and synthesis. It is rigid, readily accessible, versatile in terms of substitution patterns, and has been intensively studied by synthetic chemists over many years. The bile acids, such as cholic acid and derivatives are especially interesting. Their co-directed functionality suggests the presentation of arrays of structural units to protein surfaces, binding sites or substrates.

Now we describe a simple method for the synthesis of various cholates. A series of condensation products of cholic acid with unnatural amino acids, polyamines and peptides were prepared and tested *in vitro* for antimicrobial activity.

Chromatographic, spectral, and optical properties of the molecules have been investigated.

Influence of N-acetylcysteine on bioactivation of nitroglycerin to nitric oxide and S-nitrosothiols in the liver and brain of rats

Maria Sokołowska and **Lidia Włodek**

Institute of Medical Biochemistry, Jagiellonian University, Collegium Medicum, Kopernika 7, 31-034 Krakow, Poland

Thiols, such as glutathione and L-cysteine fulfill important, albeit not fully elucidated function in the reductive biotransformation of organic nitrates to pharmacologically active nitric oxide and S-nitrosothiols. They allow also for transport and storage of nitric oxide and act as antioxidants. One of the adverse reactions developing during the therapy with nitroglycerin and other organic nitrates is hemodynamic tolerance. To prevent development of tolerance to nitroglycerin, its doses are recommended to be increased in the course of the treatment and thiols should be administrated concurrently.

A majority of studies on the contribution of thiols to bioactivation of NTG has been conducted on the vascular endothelial cells. The aim of these investigation was to shed some light on the course and effects of the process of nitroglycerin bioactivation in the absence and presence of N-acetylcysteine in other organs, as the liver and brain of rats.

Three day nitroglycerin administration at progressively increasing doses causes a drop in the liver S-nitrosothiols and malonyldialdehyde concentrations below the control levels. It suggests that when administered in such a way nitroglycerin exhibits antioxidant

activity due to releasing the biologically active S-nitrosothiols and nitric oxide. On the other hand, in the brain, nitroglycerin does not influences S-nitrosothiols concentrations, but slightly elevates nitric oxide formation.

N-acetylcysteine given jointly with nitroglycerin substantially stimulates nitroglycerin bioactivation to the biologically active nitric oxide and S-nitrosothiols as well in the liver as in the brain. It is accompanied by a rise in non-protein thiols level and additional suppression of lipid peroxidation in hepatocytes. Therefore, is seems that the combined administration of nitroglycerin and thiols or other antioxidants is very justified not only because of their influence on the vascular epithelial cells but also on such organs as the liver and brain.

Tritium exchange and hydrogenation by spillover hydrogen of amino acids and polypeptides

Yu. A. Zolotarev[1], **A. K. Dadayan**[1], **E. M. Dorokhova**[1], **N. N. Vtyurin**[1], **V. S. Kozik**[1], **Yu. A. Borisov**[2], and **N. F. Myasoedov**[1]

[1] Institute of Molecular Genetics, Russian Academy of Sciences, Moscow, Russia

[2] Nesmeyanov Institute of Organoelement Compounds, Russian Academy of Sciences, Moscow, Russia

The study is devoted to catalytic isotope exchange and hydrogenation reactions between a solid organic compound and spillover hydrogen (SH). The reactions of high-temperature solid-state catalytic isotope exchange (*HSCIE*) and solid-state catalytic hydrogenation (*SCH*), were studied. This reactions were used for preparative production highly tritium labeled amino acids, peptides and proteins. This study utilizes *ab initio* calculations to investigate the reaction mechanism *HSCIE* and *SCH* between amino acids, peptides and spillover-tritium. The Hartree-Fock approximation and second-order Möller-Plesset perturbation theory in conjunction with 6-31G* basis sets were used to calculate potential energy surfaces for the interactions between amino acids, peptides and the model catalytic acidic center. *HSCIE* and *SCH* regio-selectivity and stereo-selectivity was examined by tritium NMR. *Ab initio* calculations were used to estimate the activation energies and structures of the transition states these reactions. The hydrogen exchange reaction occurs by a synchronous mechanism, with a transition state that is characterized by pentacoordinated carbon and three-center bond between exchanged hydrogen atoms. The *HSCIE* reaction proceeds with interaction between the exchanging H and electron-donor O and N atoms of amino acid. In the case of peptide, O and N can belong both to the same amino acid residue or to the one spatially close to it. The conformational rigidity of the peptide chain, the formation of alpha-spirals and the low three-dimensional accessibility hinder to isotope exchange. The influence of the beta-galactosidase protein from *Termoanaerobacter ethanolicus* on the exchanging ability of 1H to 3H during *HSCIE* was studied. The largest relative reactivity was found in the peptide fragment having the largest accessibility and not participating in the formation of alpha-spirals and beta-layers (KEMQKE215-220), and the smallest in the one located in the contact area between the sub-units (YLRDSE417-422). The reactivity of protein's peptide fragments depends in *HSCIE* both on their structure and on the peptide chain's accessibility for SH and mobility. The *ab initio* calculation of *HSCIE* in Ala, Gln and Ser in peptides was for the first time conducted for the protein alpha-spiral. The selected *HSCIE* conditions allowed [^{3}H]beta-galactosidase of 1440 Ci/mmol (about 50 ^{3}H per mol) to be produced with complete retention of its enzymatic activity. The conclusion was drawn that the *HSCIE* reaction can be made use of both for the production of highly tritium labeled proteins and for acquiring data on their three-dimensional structure.

Application of uniformly tritium labeled peptides in studies of organism's tissue peptidase activity

Yu. A. Zolotarev, A. K. Dadayan, E. M. Dorokhova, V. S. Kozik, and **N. F. Myasoedov**

Institute of Molecular Genetics, Russian Academy of Sciences, Moscow, Russia

An approach has been developed, using chromatographic division of a uniformly tritium labeled peptide's biodegradation products. This approach allows peptides' biodegradation under the action of the poly-enzyme system of the organism's tissue proteinases to be studied. Uniformly tritium labeled peptides were prepared using the reaction of high-temperature solid-state catalytic isotope exchange (*HSCIE*) of hydrogen for tritium[1]. Data are shown on the analysis of enkephaline hydrolysis by the poly-enzyme system of human blood plasma enkephalinases. To apply the *HSCIE* reaction, [^{3}H](Leu)-enkephalin (120 Ci/mmol) with the isotope label in all amino acid residues was produced[1]. The molar radioactivity of all its fragments was calculated according to the isotope label distribution in the peptide. 2 □Ci of [^{3}H](Leu)-enkephalin were incubated with microamounts (5 □l) of blood plasma. A mixture of 9 (Leu)-enkephalin fragments each of 5 □g was divided by HPLC with UV detection simultaneously at 250 and 280 nm[2]. The concentration of [^{3}H](Leu)-enkephalin fragments was calculated according to radioactivity in the chromatographic peaks. The enkephalin-degrading activity of aminopeptidases, dipeptidyl aminopeptidases, carboxypeptidases and dipeptidyl carboxypeptidases was simultaneously estimated in a sample. Analysis of selank (TKPRPGP) and bestatin effect on the activity of different groups of blood plasma peptidases showed bestatin to inhibit mostly aminopeptidases and carboxypeptidases and selank to be more specific to carboxy- and dipeptidyl carboxypeptidases. Thus, the use of uniformly tritium labeled high molar radioactivity peptides allows the activity of several peptidases to be simultaneously estimated in some micro amount of tissue. This approach was also used in the analysis of semax peptide (MGHFPGP) biodegradation on brain nerve cells' membranes. The main enzymes responsible for Semax biodegradation being di-peptidyl aminopeptidases leading to a successive formation of the pentapeptide HFPGP and the tripeptide PGP. The proposed method makes it possible to follow the concentration of the initial peptide and all its biodegradation products in micro amounts of tissue.

Role of H_1-receptors in the analgesic action of kyotorphin and its canavanine analogues in rats

E. Dzambazova-Maximova, A. Bocheva, and **S. Todorov**

Institute of Physiology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria

Kyotorphin (KYO) is an endogenous neuropeptide (Tyr-Arg) that may produce an opioid analgesia by release of Met-enkephalin. There are data that histamine (HA)-ergic system modulates nociception. KYO, as well as its analogues Tyr-Cav and Tyr(Cl_2)-Cav, evoked analgesia via μ-receptors or non-opiate binding sites. Nitric oxide (NO) is also involved to a different extent in the antinociceptive effects of the peptides tested.

We studied on rats the effects of the H_1-antagonist diphenhydramine (DPH) on the analgesic action of the neuropeptide KYO, and its analogues Tyr-Cav and Tyr(Cl_2)-Cav. Methylene blue (MB), a guanylate cyclase inhibitor, and L-N^G-nitroarginine ester (L-NAME), a NO synthase inhibitor, were also used. All drugs were injected intraperitoneally, and Randall-Selitto paw-pressure test was applied to test the pain-threshold changes.

DPH (100 μg/kg) induced analgesia, antagonized or reversed into hyperalgesia by NAL (1 mg/kg). DPH dose-dependently enhanced the

analgesic effects of KYO and its analogues. L-NAME alone induced a strong analgesia. Combined with KYO and its derivatives, L-NAME potentiated their antinociceptive effects after the 20^{th} min. MB abolished the analgesic effects of KYO and its analogues or even reversed them into hyperalgesic.

The present results offer strong evidence that H_1-receptors might modulate the antinociceptive action of the endogenous neuropeptide KYO and its Cav-analogues.

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