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Abstracts

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9th International Congress on Amino Acids and Proteins

Neuropeptides in Anxiety

CCK and NPY as anti-anxiety treatment targets: promises, pitfalls, and strategies

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Cholecystokinin (CCK) and neuropeptide tyrosine (NPY) can show both similar (anti-seizure) and virtually opposite (feeding, anxiety) effects. Short CCK peptides elicit panic attacks in humans and anxiogenic-like effects in some animal models, but CCK receptor antagonists have not been found clinically effective. Yet CCK overactivity appears to be involved in submissive behaviour, and CCK-B receptor expression and binding are increased in suicide victims. Preliminary data suggest that involvement of CCK and its receptor subtypes in anxiety can be better described when focusing on distinct endophenotypes, and considering environmental contingencies and confounds originating from interactions with dopamin- and glutamatergic neurotransmission. For example, CCK acting *via* both CCK-A and CCK-B receptors balances dopamine-mediated behavioural activation, which may explain why behavioural studies have produced conflicting results. Specifying the role of CCK in different brain regions along the lines of psychological dimensions, such as fear, anxiety or despair, should also lead to better understanding of its physiology. In contrast, NPY is an anti-anxiety peptide with robust effects in various animal models when given into several brain regions. NPY is known to serve as a cotransmitter in noradrenergic pathways, but its anti-anxiety effects appear independently of the functionality of this system. Studies with non-peptide antagonists selective for receptor subtypes have revealed the role of endogenous NPY in active coping. At least Y1, Y2 and Y5 receptors are involved, with the strongest evidence for contribution of Y1. Different brain regions are involved in these responses of NPY, allowing dissociation of the anti-anxiety and feeding effects.

Galanin; a novel therapeutic target for stress-related disorders?

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Galanin is a neuropeptide synthesized in brainstem norepinephrine-producing cells of the locus coeruleus and the serotonin-producing neurons of the dorsal raphe nucleus. Galanin inhibits the firing of rodent norepinephrine, serotonin and dopamine neurons and reduces their release in forebrain target regions. The distribution of galanin and its receptors and its actions on monoamine signaling has fostered interest in this neuropeptide in the field of behavioral pharmacology and the potential role of galanin in the pathophysiology of neuropsychiatry diseases such as Alzheimer's disease, epilepsy, stroke, emotional behaviors and drug addiction. In rodent models, expression of galanin in brain is increased by some, but not all, psychological stressors. Intracerebral administration of galanin in rats, or genetic modification of galanin or its receptors in mice, produces complex alterations in fear and anxiety-related behaviors that appear to be closely associated with both the site of action and the behavioral paradigm employed. Emerging evidence also demonstrates a role for galanin in mediating rodent depression-related behaviors and behavioral signs of opiate withdrawal, possibly *via* modulation of brain serotonin and norepinephrine function, respectively. While understanding of the role for galanin in modulating stress-related neural pathways and behaviors remains at an early stage, the emerging evidence from preclinical models raises the possibility of developing novel, galanin-targeting anxiolytic and antidepressant medications.

A spotlight on the role of CRF/CRF receptor systems in affective disorders

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Genetically engineered mice were originally generated to delineate the role of a specific gene product in behavioral or neuroendocrine phenotypes, rather than to produce classic animal models of depression. To learn more about the neurobiological mechanisms underlying a clinical condition such as depression, it has proven worthwhile to investigate changes in behaviors characteristic of depressed humans, such as anxiety, regardless of whether or not these alterations may also occur in other disorders besides depression. The majority of patients with mood and anxiety disorders have measurable shifts in their stress hormone regulation as reflected by elevated secretion of central and peripheral stress hormones or by altered hormonal responses to neuroendocrine challenge tests. In recent years, these alterations have been increasingly translated into testable hypotheses addressing the pathogenesis of illness. Refined molecular technologies and the creation of genetically engineered mice have allowed to specifically target individual genes involved in regulation of corticotropin releasing hormone (CRF) system elements (e.g., CRF and CRF-related peptides, their receptors, binding protein). Studies performed in such mice have complemented and extended our knowledge. The cumulative evidence makes a strong case implicating dysfunction of these systems in the pathogenesis of depression and leads us beyond the monoaminergic synapse in search of eagerly anticipated strategies to discover and develop better therapies for depression.

Vasopressin release within the brain: key to anxiety and depression?

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Rodent models are used to mimic clinically relevant endophenotypes of anxiety disorders and comorbid depression. Wistar rats bred for either high (HAB) or low (LAB) anxiety-related behavior represent divergency extremes with HAB being hyperanxious, depression-like and showing a hyper-reactivity of their HPA axis. The most typical neuroendocrine aberration associated with the HAB phenotype is the over-expression of vasopressin at the level of the hypothalamic PVN shown to be critically involved in both behavioral and neuroendocrine endophenotypes of anxiety and depression. A single nucleotide polymorphism (SNP) in the AVP promoter (A($-$40)T) causing a reduced binding of the transcriptional repressor CBF-A underlies this AVP over-expression.

CD1 mice resemble the rat model, as HAB-M are hyperanxious and depression-like in a variety of behavioral tests. Again, both behavior and the HPA axis are driven by AVP over-expression at PVN level, suggesting this phenomenon as common final pathway of anxiety and comorbid depression. Compared to HAB-M and normal CD1 controls, the LAB-M phenotype is associated with reduced expression of intra-PVN AVP which seems to underlie both dramatic non-anxiety/non-depression and signs of central diabetes insipidus. Interestingly, in this line, a non-synonymous SNP in the signal peptide (C(+4)T) is likely to result in less efficient AVP processing in the PVN and, finally, in cell death. The behavioral phenotypes can partially be rescued by antisense targeting and viral vector approaches, respectively, with HAB-M becoming less anxious and LAB-M more anxious, further confirming the key role of centrally released AVP in anxiety-related behavior.

Substance P neurotransmission: role in anxiety- and depression-related behaviour

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The tachykinin Substance P (SP) is implicated in the modulation of a variety of physiological and pathophysiological mechanisms, including stress responses, affective and anxiety-related behaviour. The distribution of SP and its preferred NK1 receptor within hypothalamic and limbic structures such as amygdala, hippocampus and septum, as well as its spatial overlap with neurotransmitter systems involved in the stress response may be the basis for an involvement in these functions. Indeed, different emotional stressors enhance the *in vivo* release of SP in distinct parts of these forebrain regions in rodents. Stress-induced SP release in areas including the amygdala and the lateral septum elicits changes in anxiety- and/or depression-related behaviour that can be reversed by specific NK1 receptor antagonists, thus underlining the functional significance of SP transmission in these areas. Preliminary evidence indicates that SP release triggered by emotional stressors is higher in animals displaying enhanced anxiety/depression-like behaviour as compared to their low anxiety/depression counterparts. The evidence of a regional hyperactive SP transmission in specific brain areas of individuals with enhanced anxiety/depression supports the original hypothesis that blockade of the NK1 receptors might have anxiolytic as well as antidepressant effects in patients with depression and/or anxiety disorders. The finding that existing antidepressants, considered as first line treatment for both depression and most anxiety disorders, do not change the expression of NK1 receptors or the number of SP binding sites in the brain supports the notion that NK1 receptor blockade represents a radical new approach to the management of these stress-associated disorders.

Oxidative Stress and Protein Modification by Reactive Oxygen and Nitrogen Species

Protein-lipid interactions: membrane structure, lipid composition, and the effect of oxidants and tocopherols

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An enormous selection of proteins and enzymes have, at some point in their mode of action, a dependency on lipids as substrates or ligands, as modifying or allosteric agents, or as the components of membranes on which they act or are embedded. Many of these interactions have been well described, as in the case of lipid transfer proteins responsible for the intracellular movement of fatty acids, phospholipids, and sterols. But even in these cases the mechanism of lipid transfer to membranes of differing composition remains largely opaque.

The tocopherols (vitamin E) have long been recognized as hydrogen atom donors that inhibit lipid peroxidation and, thus, as the dominant lipid-soluble antioxidants. In the past fifteen years or so this perception has begun to change as other roles for tocopherols have been discovered that appear not to depend on their action as antioxidants. Intriguingly, not all tocopherols share the same profile of activities; α-tocopherol appears to inhibit PKC by an indirect means, γ-tocopherol has growth inhibitory effects on selected cancer cell lines by an unknown mechanism, and δ-tocopherol is cytotoxic to certain cells. This talk will present an overview of the membrane biochemistry of the tocopherols, their contribution to the modification of lipid-dependent proteins and enzymes, and some new tools (molecular probes and spectroscopies) that are available for studies in this area.

Evaluation of plasma thiols redox status; facts and artifacts

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The topic of plasma thiols is very important and complex, and still undecifered. There are both reduced and oxidized forms of main thiols, cysteine, cysteinylglycine, glutathione, homocysteine and mercaptoalbumin, in human plasma. Reduced forms are characterized by a free sulfhydryl groups. Oxidized forms exist as disulfides which include free oxidized forms – symmetrical and unsymmetrical disulfides of low molecular-mass thiols, and mixed disulfides with proteins, mostly albumin. All these reduced and oxidized forms in plasma interact *via* redox and disulfide exchange reactions, and comprise a dynamic system referred to as thiol redox status. The thiol redox status of intracellular and extracellular compartments is critical in the determination of protein structure, regulation of enzyme activity, and control of transcription factor activity and binding. Because of the importance of thiols and disulfides and the possible use of their plasma levels as biomarkers of health status, there is a need to fully understand relationship between all of the thiols and disulfides components. The measurement of thiols and disulfides in plasma has proven to be difficult. Most thiols are present in low concentration and are very unstable in the isolated plasma. Because of this problem, few methods for the accurate measurement of thiols and disulfides in plasma have been described. Results of the measurement can be affected by the following: (1) oxidation of thiols in acidified

samples during acid deproteinization, (2) oxidation after restoring neutral or alkaline pH, (3) shift in the thiol/disulfide equilibrium because of irreversible blocking of sulfhydryl groups, and during acid deproteinization, (4) reaction of electrophilic sulfhydryl group blockers with amino groups. In this presentation most frequent pitfalls in thiols and disulfides measurements in plasma will be identified, with particular emphasis to the high-performance liquid chromatographic procedures.

Mechanisms of redox signaling; from nitric oxide to oxidized low density lipoproteins

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Cellular redox signaling is mediated by the post-translational modification of proteins by reactive oxygen/nitrogen species or the products derived from their reactions. Nitric oxide is perhaps the best understood in this regard with two important modifications of proteins known to induce conformational changes leading to modulation of function. The first of these is the nitrosylation of metalloproteins by NO primarily at ferrous heme centers. The two examples for which this mode of action has been defined are soluble guanylate cyclase and the newly discovered NO-cytochrome c oxidase signaling pathway in mitochondria. The second mechanism is through the conversion of NO to species such as peroxynitrite that can lead to activation of signaling pathways in the vasculature in response to the flow-dependent forces (shear stress) and exposure to oxidized lipids including those found in oxidized low density lipoprotein. The pathways involved include the activation of the MAP kinases and adaptive cytoprotective mechanisms. In endothelial cells these processes involve an interaction with the mitochondrion in transducing these signals. The insights gained from our understanding of the biology of both of these mechanisms will be discussed in the context of cardiovascular disease.

Cadmium signalling in sunflower. The involvement of superoxide anion and H_2O_2

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Reactive oxygen species (ROS), such as superoxide anion ($O_2^{\cdot-}$) and H_2O_2, could function as intracellular second messengers. In the present work, we studied the participation of $O_2^{\cdot-}$ and H_2O_2 in the signal transduction pathway that mediates Cd^{2+} toxicity symptoms in sunflower leaf discs, by using several inhibitors ($LaCl_3$, ODQ, DPI, EGTA, Zn^{2+}) or inducers (Ca^{2+}). H_2O_2 and $O_2^{\cdot-}$ were evidenced by nitroblue tetrazolium (NBT) and diaminobenzidine assays. Total GSH decreased significantly after 14 h of 0.5 mM Cd^{2+} treatment and the effect was partially reversed by a Ca^{2+} channel blocker, $LaCl_3$, and by ODQ, an inhibitor of cGMP. TBARS content increased an average of 30% under Cd^{2+} stress and this increase was not reversed by any of the inhibitors tested. Catalase activity was reduced by Cd^{2+} and this decay was partially reversed by $LaCl_3$, Ca^{2+} and Zn^{2+}. The production of $O_2^{\cdot-}$ was completely inhibited by 0.5 mM Cd^{2+} after 14 h of treatment. Again, $LaCl_3$ abolished the effect of Cd^{2+}. However, when Cd^{2+} was used at 0.01, 0.1 or 0.5 mM for 1 h, $O_2^{\cdot-}$ formation in treated discs occurred as in controls. The inhibition of the $O_2^{\cdot-}$ formation started after 3 h of treatment with 0.5 mM Cd^{2+} or later with the lower Cd^{2+} concentrations. The results obtained in relation to SOD activity or H_2O_2 formation were not clear enough to definitively clarify their roles in Cd^{2+} toxicity. The role of NADPH oxidase-like enzyme as the target of Cd^{2+} and the involvement of Ca^{2+} as mediator of Cd^{2+} effect is discussed.

Mechanism and consequences of protein *N*-homocysteinylation in humans

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In humans homocysteine (Hcy) is formed from dietary methionine as a by-product of biological methylation reactions. Elevated levels of Hcy are thought to contribute to the pathophysiology of coronary heart disease and stroke in humans. Our hypothesis is that metabolic conversion of Hcy to Hcy-thiolactone by methionyl-tRNA synthetase initiates a pathway that underlies Hcy toxicity in humans. Subsequent steps in the pathway include protein *N*-homocysteinylation by Hcy-thiolactone, induction of an autoimmune response by *N*-Hcy-protein, which in turn leads to damage to the vascular wall, a hallmark of atherosclerosis. Protein *N*-homocysteinylation occurs spontaneously and is due to an intrinsic ability of Hcy-thiolactone to form *N*-Hcy-protein adducts in which a carboxyl group of Hcy is *N*-linked to ε-amino group of a protein lysine residue. Protein *N*-linked Hcy, present in each blood protein examined, constitutes a significant pool of Hcy in the blood. Because *N*-homocysteinylation causes protein damage and increases protein's susceptibility to further oxidative damage, *N*-Hcy proteins are toxic to cells and are recognized as neo-self antigens. Indeed, auto-antibodies, which specifically recognize an *N*ε-Hcy-Lys epitope on *N*-Hcy-proteins, occur in humans. Serum levels of the anti-*N*-Hcy-protein auto-antibodies positively correlate with plasma total Hcy, but not with plasma cysteine or methionine levels. In groups of male patients with stroke or coronary artery disease, levels of anti-*N*-Hcy-protein auto-antibodies and tHcy are significantly higher than in control groups of healthy subjects. Our findings show that the formation of *N*-Hcy-proteins has important physiological consequences and support a hypothesis that *N*-Hcy-protein is a neo-self antigen, which contributes to immune activation, an important modulator of atherogenesis.

Alteration of cellular redox-dependent signaling under development of K562 cells resistance to doxorubicin

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Redox-dependent character of formation of cancer cells resistance to doxorubicin (DOX) is actual for research of mechanisms of multidrug resistance caused by its pro-oxidant effect and widespread use for tumor chemotherapy. Development of resistance of human erythroleukemia K562 cells to DOX has been shown to be associated with a rise of adaptive antioxidant response included an increase of antioxidant enzymes (Cu, Zn-SOD, Mn-SOD) and GSH-related enzymes activities as well as suppression of ROS production through a decrease of DOX-activated enzymes. The decrease of ROS production correlated with suppression of total PTK activity and significant changes of redox-dependent messengers glutaredoxin (Grx) and thioredoxin (Trx) activities and mRNA levels. High growth of Grx1 mRNA level was found in K562/DOX cells in comparison to its slightly detected level in sensitive cells. In contrast low growth of Trx1 mRNA level was observed in resistant cells. Redox-dependent alteration of PTK activities, activities and expression of Grx1 and Trx1 has been suggested to play important role in the rise of adaptive antioxidant response under development of K562 cells resistance to DOX.

Antioxidant protection and changes of thioredoxin and glutaredoxin relation under eltacin treatment of patients with obstructive bronchitis

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Redox-dependent development of human chronic obstructive lung diseases (chronic obstructive brochitis, lung emphysema) depends on the risk factor caused by enhancement of cellular ROS production. The treatment of old patients (67.7 ± 3.2 years old) by metabolic drug eltacin resulted in the intensive regulation of GSH level and activities of GSH-dependent enzymes, Cu, Zn-superoxide dismutase, catalase upto normal values in erythrocytes. The effect of eltacin was associated with the decrease of lipid peroxidation (upto 1.5–1.8 fold) due to the decrease of ROS ($O_2^{\cdot-}$, H_2O_2) level. The opposite change of relationship between ROS-signaling messengers thioredoxin and glutaredoxin was observed in erythrocytes and plasma of patients as well as positive correlation between glutaredoxin activity and GSH level. The growth of antioxidant status was correlated with positive alteration of patients states: elevation of peak flow, SaO_2, pO_2 levels and decreasing of CO_2, alteration of circadian rhythms of arteriotony to normal standard. The mechanism of eltacin action is connected with the regulation of redox status owing to elevation of GSH maintenance. It may be concluded that the antioxidative properties of eltacin give it perspective for the use in the therapy of chronic obstructive brochitis. Gerontological effect of eltacin in prediction, prevention and treatment of inflammatory diseases connected with aged and raised under environmental pollutions is discussed.

Influence of γ-glutamyltranspeptidase inhibitors on the cytotoxicity of doxorubicin and cis-platinum in HepG2 cells

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A risk of the damage to normal tissues is a particular problem in clinical chemo- and radiotherapy of cancer as it significantly limits acceptable doses of drugs and radiation. Toxicity of drugs and radiation in the cells is largely dependent on the level of thiols. High glutathione level and elevated activity of γ-glutamyltranspeptidase (γGT) are characteristic features of tumor cells. In the present studies, in order to increase efficacy of cytostatics, an attempt has been made to modulate γGT activity in human hepatoblastoma (HepG2) cells. We expected that administration of γGT inhibitors, acivicin (AC) and 1,2,3,4-tetrahydroisoquinoline (TIQ) may additionally enhance cytostatic action of doxorubicin and cis-platinum. Our previous research revealed that TIQ inhibited γGT activity in different brain structures, besides it also turned out to be an inhibitor of the isolated enzyme.

Cytotoxicity of the tested compounds was determined by MTT assay which measures metabolic integrity by means of the leakage of LDH.

The obtained results showed that the above-mentioned γGT inhibitors introduced to the medium revealed cytotoxic effect on their own, which was accompanied by the increased glutathione level in the cells. Doxorubicin or cis-platinum increased cytotoxicity approx. 30% and 60% in relation to the control, and both inhibitors drastically deepened the cis-platinum and doxorubicin cytotoxicity. Simultaneously, as well doxorubicin as cis-platinum radically decreased GSH levels, and γGT inhibitors remained without any effect. Therefore, the obtained results confirm that the inhibitors of γGT can enhance pharmacological action of doxorubicin and cis-platinum, that may permit to decrease doses of drugs and concomitantly their side effects. This indicates that research into application of γGT inhibitors in cancer therapy and search for new compounds possessing such activity can contribute as well to reduction of anticancer drug toxicity as to elevation of their efficacy.

Proteomics analysis of oxidized lipid-protein adducts; insights into mechanisms of adaptation and induction of apoptosis

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The formation of lipid oxidation products through either enzymatic or non-specific lipid peroxidation generates broad spectrum of structurally diverse compounds. Initially it was thought these products have limited specificity in their ability to elicit a biological response. With the advent of advanced proteomics techniques this perspective has changed. One such class of products, the electrophilic lipids, are now being recognized as important mediators of physiological and pathological processes. These lipids modify proteins due to the presence of an electrophilic carbon center, and may also contain other reactive groups such as aldehydes. The effect that oxidized lipids have on cell signaling is dependent on the concentration of the lipid. At low concentrations, adaptation to oxidative stress in the vasculature appears to be mediated by induction of antioxidant defences including the synthesis of the intracellular antioxidant glutathione. Recent studies have revealed that the mechanism through which the electrophilic lipids orchestrate these adaptive responses in the vasculature involves regulation of genes controlled by the antioxidant response element. Interestingly, higher concentrations of these oxidized lipids induce apoptosis through mechanisms that have yet to be defined in detail. Using a proteomics approach with tagged electrophilic lipids, we have identified a subset of proteins which we have termed the electrophile responsive proteome. The identity of these proteins will be discussed in the context of our increasing understanding of redox signaling and the response of the cell to oxidative stress.

Cadmium-induced oxidative damage is reduced by nitric oxide in sunflower plants

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Cadmium (Cd) is a heavy metal present in soils, toxic for humans, animals and plants at very low concentrations. Nitric oxide is a bioactive free radical involved in many physiological responses. Cadmium toxicity and NO protection were evaluated in relation to some antioxidant parameters, as soluble and enzymatic antioxidants, in sunflower leaves, after 10 days of treatment with 0.5 mM Cd_2Cl, in plants pretreated for 7 days with 100 μM SNP (as NO donor). Plant growth (DW) was significantly reduced by Cd and was recovered in plants pretreated with NO. Chlorophyll content decreased to 48% and TBARS increased 33% over the controls, but NO returned their contents to 88% and 100% of the C. Ascorbate peroxidase (APOX) and superoxide dismutase (SOD) activities increased significantly with Cd, while CAT and GPOX activities decreased. Glutathione (GSH) content was lower and ascorbic acid (AsA) content was higher than controls in Cd-treated plants. Nitric oxide pretreatment alone did not modify the studied parameters respect to the control, except for APOX activity and AsA content, which increased even in the absence of the metal. SOD activity was reduced to C levels, CAT and GPOX activities were returned to values close to the controls,

while GSH decline was reverted by NO pretreatment. In the present work, we present evidence of the protection exerted by NO against the oxidative stress and toxicity induced by Cd on sunflower leaves.

An important role of sulfane sulfur at a catalytic cysteine of rat mercaptopyruvate sulfurtransferase in defense against oxidative stress

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In the catalytic process of transsulfuration of rat mercaptopyruvate sulfurtransferase (MST, EC 2.8.1.2), stable sulfane sulfur is formed at the catalytic site, Cys^{247} as a reaction intermediate. However, the physiological role of the stable sulfane sulfur remains unknown. In this study, we hypothesized that this sulfane sulfur protected MST from inactivation by oxidative stress. We obtained recombinant rat MST according to a modified method by Nagahara and Nishino (J. Biol. Chem. 1996, 271, 27395). To form sulfane sulfur at Cys^{247}, MST was incubated with an excess molar dose of thiosulfate on ice for 40 min. The mixture was gel-filtered to remove excess thiosulfate. In oxidation experiments, the sulfane sulfur-containing MST was incubated with a 1, 2, 5 and 50-fold molar dose of hydrogen peroxide on ice for 20 min. After gel-filtration to remove excess oxidant, the oxidized MST was incubated with an excess molar dose of DTT on ice for 20 min. The rhodanese activity of each sample was assayed. Control MST without formation of sulfane sulfur was inhibited by a stoichiometric concentration of hydrogen peroxide, and DTT restored the activity. Further, an excess molar dose of hydrogen peroxide inactivated MST. However, sulfane sulfur-containing MST resisted oxidative stress from inactivation; even after the MST was exposed by a 50-fold molar dose of hydrogen peroxide, about 20% of the control enzyme (un-oxidized MST) activity remained, and the activity was restored to about 60% by DTT treatment.

Differential requirements of calcium for oxoglutarate dehydrogenase and mitochondrial nitric-oxide synthase under hypoxia: impact on the regulation of mitochondrial oxygen consumption

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Studies with isolated mitochondria are performed at artificially high pO_2 (220 to 250 μM oxygen), although this condition is hyperoxic for these organelles. It was the aim of this study to evaluate the effect of hypoxia (20–30 μM) on the calcium-dependent activation of 2-oxoglutarate dehydrogenase (or 2-ketoglutarate dehydrogenase; OGDH) and mitochondrial nitric-oxide synthase (mtNOS). Mitochondria had a P/O value 15% higher in hypoxia than that in normoxia, indicating that oxidative phosphorylation and electron transfer were more efficiently coupled, whereas the intramitochondrial free calcium concentrations were higher (2–3-fold) at lower pO_2. These increases were abrogated by ruthenium red indicating that the higher uptake *via* the calcium uniporter was involved in this process. Mitochondria at high calcium concentration microdomains may produce nitric oxide, given the $K_{0.5}$ of calcium for OGDH (0.16 μM) and mtNOS ($\sim$1 μM). Nitric oxide, by binding to cytochrome oxidase in competition with oxygen, decreases the rate of oxygen consumption. This condition is highly beneficial for the following reasons: i, these mitochondria are still able to produce ATP and support calcium clearance; ii, it prevents the accumulation of ROS by slowing the rate of oxygen consumption (hence ROS production); iii, the onset of anoxia is delayed, allowing oxygen to diffuse back to these sites, thereby ameliorating the oxygen gradient between regions of high and low calcium concentration. In this way, oxygen depletion at the latter sites is prevented. This, in turn, assures continued aerobic metabolism which may involve the activated dehydrogenases.

Genetics

Analyzing signaling complexes: networking for drug discovery

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Chronic inflammation is a pathogenic factor in a wide range of therapeutic areas, such as Rheumatoid Arthritis (RA), psoriasis and Crohn's Disease. For several of these severe chronic inflammatory conditions there is unmet medical need. Among other cytokines Tumor Necrosis Factor (TNF)-α is the key mediator driving inflammation. Current treatments for RA and psoriasis are based on protein therapeutics, including soluble receptors and antibodies. However, no small molecule drugs have been approved yet.

To identify and prioritize novel therapeutic targets we have used a systematic large scale functional proteomics approach mapping the TNF-α signal transduction pathway. We applied tandem affinity purification and mass spectrometry (TAP-MS) to identify crucial players in the protein–protein interaction network. Several interactors were found to modulate the pro-inflammatory response and therefore represent novel opportunities for therapeutic intervention in inflammatory diseases. Moreover, through a chemical proteomics approach it has become feasible to map active chemical compounds back on the pathways by identifying the protein interactors of the immobilized compounds. By using both functional and chemical proteomics the mode-of-action of pathologically-relevant proteins plus novel and existing drugs can thus be determined. Such a systems biology approach can be applied to any signal transduction pathway involved in disease and translated into novel therapeutic and diagnostic approaches.

Molecular biology of tuberous sclerosis: tuberin regulates the cyclin-dependent kinase inhibitor p27

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TSC1, encoding hamartin, and TSC2, encoding tuberin are tumor suppressor genes responsible for the autosomal dominantly inherited disease tuberous sclerosis (TSC). TSC affects $\sim$1 in 6000 individuals and is characterized by the development of tumors, named hamartomas, in different organs. Hamartin and tuberin form a complex, of which tuberin is assumed to be the functional component. The TSC proteins have been implicated in the control of cell cycle and cell size.

The ubiquitin-proteasome proteolytic pathway is responsible for the highly selective turnover of cellular proteins. Skp2 is the F-box protein, which together with other proteins, form an SCF (Skp1/cullin/F-box

protein)-type E3 ubiquitin ligase complex whose task is to target the cyclin-dependent kinase inhibitor p27 for degradation by the proteasome. Recently, we found that tuberin binds p27 and negatively regulates its interaction with Skp2. Here, our new finding that tuberin also regulates the nuclear localization of p27 throughout the cell cycle will be presented and discussed.

Biogenesis of protein phosphatase 2A (PP2A)

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Protein serine/threonine phosphatases (PSTPs) are multisubunit enzymes consisting of catalytic and substrate-targeting subunits. A prime example for the PSTPs multisubunit architecture is protein phosphatase 2A (PP2A). The majority of intracellular PP2A consists of a catalytic C subunit associated with a regulatory A subunit that serves as a scaffold for the interaction with one of several substrate targeting B-type subunits. Malfunctions of PP2A are involved in the genesis of human diseases from cancer to Alzheimer disease.

More recently we identified a novel and phylogenetically conserved mechanism of how PP2A acquires its intracellular specificity and activity for serine-/threonine-phosphorylated substrates. We now propose a model of PP2A biogenesis, which for the first time integrates the generation of catalytically active C subunit with holoenzyme assembly. A detailed understanding of PP2A biogenesis will help to elucidate the pathogenesis of the aforementioned diseases and should lead to the development of novel therapeutic concepts.

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Chromatin and cancer – the role of histone deacetylases

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Histone deacetylases (HDACs) are important regulators of chromatin structure and gene expression. By increasing the attraction between DNA and histones, HDACs mediate the compaction of chromatin thereby reducing the accessibility of specific genes for the transcription machinery. Inhibitors of HDACs such as butyrate or trichostatin A have been shown to induce cell cycle arrest, apoptosis or cell differentiation in human tumor cells and animal models. Therefore, HDAC inhibitors are currently tested as anti-cancer agents in clinical trials. However due to the presence of more than a dozen HDACs in mammalian cells the identity of the relevant HDACs as targets for tumor drugs is still unclear.

We have studied the function of the first identified deacetylase HDAC1 by knocking out the HDAC1 gene in mice. HDAC1 deficient mice die during embryogenesis due to developmental problems and proliferation defects. Similarly, siRNA-mediated silencing of HDAC1 in human tumor cells leads to apoptosis. A specific set of tumor suppressors is up-regulated in the absence of HDAC1 in both murine and human cells. Finally, teratocarcinomas derived from HDAC1-deficient embryonic stem cells show reduced size due to increased apoptosis. Our results indicate that HDAC1 is a relevant target for tumor drugs.

From allergen genes to allergy vaccines

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Immunoglobulin E (IgE)-mediated allergy affects more than 25% of the population in industrialized countries. During the last years the cDNAs coding for most of the relevant disease-eliciting allergens have been isolated and expressed as recombinant allergens. Based on recombinant allergens it has become possible to reconstruct the epitope complexity of the most common allergen sources and novel diagnostic tests have been developed which allow the dissection of patients reactivity profiles down to the single molecules. Furthermore it has become possible to develop by recombinant DNA technology a new type of allergy vaccines with reduced allergenic activity. The engineering of hypoallergenic derivatives of the major birch pollen allergen, Bet v 1, by genetic engineering and the vaccination of birch pollen allergic patients ($n = 124$) in a double-blind, placebo-controlled study will be reported. Active treatment induced protective IgG antibodies which inhibited allergen-induced release of inflammatory mediators. Furthermore a reduction of cutaneous sensitivity as well as an improvement of symptoms in actively treated patients was observed. Most important, rises of allergen-specific IgE induced by seasonal birch pollen exposure were significantly reduced in vaccinated patients. Thus, a novel allergy vaccine based on genetically engineered allergen derivatives was developed which not only ameliorated allergic reactions, but also reduced the IgE production underlying the disease. According to this proof of concept study it can be envisioned, that it will be possible to develop therapeutic and prophylactic vaccines against the most common forms of IgE-mediated allergies.

Medicinal Chemistry

Does L tyrosine modulate any of the adverse effects of phinobarbitone on the biochemical systems in adult male rats?

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This study aimed at elucidation of the modulating action of L tyrosine [15/kgb.w.] on phinoba rbitone [10 mg/kgb.w.] induced adverse effects on the enzymatic and hormonal pathways in adult male rats.

Erthyrocyte glutathione peroxidase [GPX] and superoxide dismutase [SOD] activities beside the concentration of plasma nitric oxide [NO] were assayed. Liver functions [AST, ALT, alkaline phosphatase, total protein, albumen and total bilirubin], thyroid functions [t4, t3 and tSH] as well as LDH and CK were also estimated. Additionally, pituitary-adrenal axis (ACTH an corticostereone); kidney functions (urea and creatinine) and testicular functions (testirone and acid phosphatase) were determined.

Results: The obained data revealed that while L tyrosine could high significantly raise GPX and SOD levels and reduce the level of NO in adult male rats, phenobarbitone, on the contrary significantly decreased the levels of these enzymes and significantly raised the levels of both enzymes although they could not restore baseline levels, while the level of NO markedly attentued to near baseline level. Each of L tyrosine and phenobarbitone either in separate or in successive application could significantly increase AST ana alkaline phosphatase activities and also markedly raised the ALT activity, while high significantly decreased each of serum total protein, albumin and bilirubin levels as compared to the control. L tyrosine significantly owered serum T4 and T3 levels and raised serum TSH level as compared to the control. Each of the two reagents either in separate or in successive application could significantly raise serum LDH and CK activities and also significantly raised plasma ACTH and coticosterone levels as compared to the control. Each of L tyrosine phenobarbitone affected renal function, as they could significantly increase serum urea and creatinine levels as compared to the control. Each of the two drugs significantly reduced serum testosterone level, while significantly raised serum acid phosphatase activity in all treated groups as compared to the control.

Conclusion: Noteworthy, treatment with L tyrosine to injection with phinobarbitone markedly modulated the decrease in GPX and SOD activities and the increase in the NO level caused by phenobarbitone. Each of L tyrosine and phenobarbitone either in seoarate or in successive application affected the functions of the most vital organs of adult male rats.

In vitro effects of excess amino acids on *Chlamydia trachomatis* and *Chlamydia* (*Chlamydophila*) *pneumoniae*: a comparative study

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Effects of single additions of amino acids on the growth of *Chlamydia trachomatis* were investigated and compared with those on *C.* (*Chlamydophila*) *pneumoniae*. Some essential amino acids, administered at the middle of infection course, produced varying degrees of abnormalities in the development of both species. These effects were more prominent when chemicals were introduced as early as 2 h post-infection. Most effective amino acids on *C. trachomatis* were leucine, isoleucine, methionine and phenylalanine. These chemicals elicited similar activities against *C. pneumoniae*, except methionine that was, surprisingly, less suppressive to its infection. Tryptophan or valine marginally inhibited *C. trachomatis* growth and, paradoxically, led to a considerable enhancement of *C. pneumoniae* growth. On the other hand, some nonessential amino acids administered at the middle or throughout the infection period differentially affected the development of both species. Of importance, glycine and serine efficiently inhibited *C. trachomatis* growth. However, *C. pneumoniae* was relatively less sensitive to these agents. Another difference was demonstrated by glutamate, glutamine, and aspartate, which stimulated *C. pneumoniae* growth more than that of *C. trachomatis*. Overall, some conspicuous differences in susceptibilities to the increase in amino acid levels were revealed using two isolates, representing *C. trachomatis* and *C. pneumoniae*. This study could serve as a basis to examine if there are distinctive patterns of sensitivities to amino acid overload among different chlamydial species. Perturbation in levels of amino acids, i.e. leucine and isoleucine, could be a new method to help treat or prevent chlamydial diseases.

Calcium intake dependent changes of brain proteins GAP-43, BASP1 and MARCKS in spontaneously hypertensive rats

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It is well known that genetically conditioned hypertension in rats is accompanied by significant learning deficiency. Present study was designed to evaluate calcium supplement-dependent changes in the axonal termini of spontaneously hypertensive rats (SHR). We investigated calcium input dependence of content and structural changes of learning related brain proteins GAP-43, BASP1 and MARCKS in SHR**.** The experiments were carried out with brains of male (P18) low calcium input SHR (systolic pressure 175 ± 9 mmHg), high calcium input SHR (systolic pressure 160 ± 9 mmHg), which have received supplementary calcium ($CaCl_2$) in drinking water, and normotensive Wistar-Kyoto rats (WKY, systolic pressure 130 ± 6 mmHg). In low calcium input SHR synaptosomes, BASP1 and MARCKS concentrations were essentially lower, than in WKY ones. High calcium input increased BASP1 and MARCKS concentrations in SHR synaptosomes, but not up to WKY level. Synaptosomal GAP-43 concentrations were also dramatically different in these three groups. In low calcium input SHR synaptosomes GAP-43 was nearly absent, while its proteolytic fragment GAP-43-3 (lacking 40 N-terminal amino acids) was detected in high concentration. Intact GAP-43 was detected in high calcium input SHR, but it was lower, then in WKY group. It was shown earlier that GAP-43-3 is produced by GAP-43 cleavage at Ser41 by calcium-dependent protease calpain. Prevalence of GAP-43-3 over intact GAP-43 can be explained by increased intracellular calcium characteristic for SHR. Our data confirm that the both spontaneous hypertension and brain protein abnormalities in SHR are caused by impairment of calcium metabolism.

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Microalbuminuria in children with vesicoureteral reflux

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Vesicoureteral reflux (VUR) is a common congenital anomaly of the urinary tract that may be inherited. Reflux of infected urine may cause scarring in susceptible kidneys, with the potential for compromise of renal function. The aim of the study was to evaluate the eventual influence of different grades of VUR on glomerular damage using microalbuminuria as a parameter.

Children with VUR (n: 35, aged 1 month–16 years) detected by voiding cystourethrography (VCUG) were investigated. According to a grade of VUR, patients were separated into three groups. The first group included 12 children with VUR grade I–II. The second group was consisted of 12 children with grade III of VUR. Patients with VUR grade IV–V (n: 11) were members of the third group. Control group was consisted of 17 healthy children (aged 1–16 years). Microalbuminuria was examined in samples of morning urine specimens using Microalbumin/Creatinine reagent kit. Serum urea, creatinine levels and creatinine clearance (CCR) were measured as markers of renal function.

The mean value of microalbuminuria in the third group showed statistically significant increase ($p < 0.05$) compared to control values (12.45 ± 12.28 vs. 6.29 ± 2.82 mg/dl).

CCR in the third group was $98.37 \pm 24.56\,ml/min/1.73\,m^2$, which was statistically significant decrease ($p < 0.05$) in comparison to a group of healthy children (128.81 ± 21.12). There were no statistically significant changes of microalbuminuria and CCR in the first and second group compared to control values.

We discussed increase of microalbuminuria and decrease of CCR in children with high grade of VUR (the third group) as a possible consequence of retrograde urine flow (intrarenal reflux), glomerulosclerosis and consecutive hyper filtration.

RGD-peptides and their mimetics as the inhibitors of Mycobacteria into the cells invasion

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The Mycobacteria invasion into the cells depends on the formation of the complex between bacterial antigen Ag85 and extracellular matrix protein, fibronectin. The interaction of fibronectin with cellular receptors of integrin type enables the entry of bacteria into the host cells. The RGD-peptides, which are known as inhibitors of fibronectin – intergrin receptor interaction, can be therefore used as inhibitors of Mycobacteria phagocytosis. Basing on this idea we examined several series of RGD-peptides and their analogues as potential phagocytosis inhibitors. They were related to the peptide sequences of different regulatory proteins containing RGD-moiety. The presence of charged groups of Arg and Asp, located on the proper distance is crucial for the activity. The charged groups must be situated nearly coplanar, and at the distance of about 10 Å. Such a conclusion was confirmed by us by the study of series of compounds of Arg-$(Gly)_n$-Asp type. A new type of integrin inhibitors: ω-guanidino-carboxylic acids, was discovered. The activity appears when the hydrocarbon chain length becomes that of guanidinohexanoic acid. The α-amidinoamino acids from derived of proteinoceous amino acids were as a rule without inhibitory activity.

Influence of L-canavanine on analgesic effects of amino acids L-arginine, L-ornithine and L-citrulline in rats

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L-Canavanine (L-Cav) is a potent antimetabolite and structural analog of L-Arginine, produced by legumes such as jack bean, *Canavalia Ensiformis* and exerted a strong antinociceptive effect. It is known that L-Cav is an inhibitor of iNOS.

The purpose of the present study was to investigate the influences of L-Cav on the antinociceptive effects of L-arginine (L-Arg), (L-Orn), (L-Citr). The changes in the nociceptive effects were examined on male Wistar rats in acute pain by paw-pressure (PP), hot plate (HP) and tail-flick (TF) tests using an analgesiameters (Ugo Basile). L-Cav, L-Arg, L-Orn and L-Citr were administered intracerebroventricullary (i.c.v.) at a dose $20\,\mu g/20\,\mu l$.

L-Cav significantly reversed the pain thresholds of L-Arg, L-Orn and L-Citr into hyperalgesia in PP test. In HP test application of L-Cav significantly decreased the analgesic effects of both L-Arg and L-Orn, but not L-Citr. L-Cav decreased TF latency on L-Orn and significantly reversed the analgesic effects of L-Arg and L-Citr into hyperalgesia.

These results show that in the mechanical and thermal nociception tests L-Cav influences the analgesic effects of L-Orn, L-Arg, L-Citr in different way in rats.

Problems, pitfalls and presentation of amino acid data

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The clinical 'aminogram' is a very complex piece of information and the analytical process involved is time consuming and expensive. Unfortunately, the results obtained are often compromised by lack of relevant clinical information, lack of dietary and medication information and poor specimen quality. Additionally, the typical practice of simply reporting a set of numerical values potentially restricts the yield of information and is poorly interpreted by clinicians. The intelligent use of reference intervals is also difficult when dietary information is absent.

Techniques such as ratios, two-dimensional plots and other decision support algorithms, if used in an informed way can compensate for many of the problems cited above and enable the 'fingerprinting' of amino acid patterns. The reliance on absolute reference intervals is also overcome. This approach can reveal results that, although not abnormal in absolute terms, may be anomalous relative to the overall pattern.

When assessed using these techniques the quantitative amino acid profile may permit assessment of nutritional status, indication of secondary disorders and detection of mildly expressed inherited disease.

CSF phenylalanine – tyrosine ratio: a pointer to defects in pterin metabolism?

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Tetrahydrobiopterin (BH4) is an essential cofactor for hydroxylases catalysing the formation of precursors of neurotransmitter synthesis. Disorders of BH4 synthesis result in a deficiency of brain dopamine and serotonin synthesis and neurological dysfunction, including dystonia. These hydroxylases include phenylalanine (phe) hydroxylase and autosomal recessive disorders of BH4 metabolism can be identified by hyperphenylalaninaemia and an elevated phenylalanine to tyrosine (tyr) (P/T) ratio. In contrast, patients with dystonia arising from autosomal dominant mutations in GTP cyclohydrolase (the first committed step of BH4 biosynthesis) invariably have a normal plasma phenylalanine concentration and P/T ratio. Initial identification of these patients often requires a phenylalanine loading test to be performed in order to demonstrate the partial BH4 deficiency state.

However, in patients with dystonia suspected to arise from autosomal dominant GTP cyclohydrolase deficiency, we observed that the CSF P/T ratio exceeded that found in control samples.

In control patients ($n = 39$) without demonstrable metabolic disease CSF phe, tyr and P/T ratio were in the range: phe 3–12 μmol/L; tyr 3–9 μmol/L and P/T 0.29–1.59 (mean $\pm$ 2 SD: 0.16–1.48) and the ratio was independent of age. In patients with the suspected BH4 deficiency values for CSF phe and tyr were within the normal range, however these patients showed an increased CSF P/T ratio in the range 1.45–3.38.

These results suggest that the CSF P/T ratio may be a useful initial pointer to defects in BH4 metabolism where the deficiency is either not expressed in the liver or sufficiently to cause overt perturbation of phenylalanine metabolism.

Plasma arginine correlations in trauma and sepsis

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Arginine (ARG) is an amino acid (AA) with unique properties and with a key-role in the metabolic, immune and reparative response to trauma and sepsis. This study has been performed to characterize the correlations between plasma levels of ARG, of other AA and of multiple metabolic variables in trauma and sepsis.

Two-hundred and sixty-three plasma amino-acidograms with a large series of additional biochemical and blood variables were obtained consecutively in 9 trauma patients who developed sepsis, undergoing total parenteral nutrition with dextrose, fat and a mixed AA solution containing 10.4% arginine.

ARG was low soon after trauma, then it increased with increasing distance from trauma and with the development of sepsis. ARG was also directly related to the AA infusion rate (AAIR) and for any given AAIR, was lower after trauma than after the development of sepsis. ARG was also related directly to the plasma levels of most of the other AA, the best correlation being that with lysine ($r^2 = 0.81$, $p < 0.001$). These correlations were often shifted downwards (showing lower ARG for any given level of the other AA) in measurements performed after trauma, compared to those performed after development of sepsis; this effect was more pronounced for the correlations with branched chain AA. Correlations between ARG and non-AA variables were not particularly relevant. The best simultaneous correlates of ARG, among variables involved in plasma ARG availability, were citrulline level, AAIR and urinary 3-methylhistidine excretion (accounting for the effect of endogenous proteolysis) (multiple $r^2 = 0.70$, $p < 0.001$). Plasma ornithine (ORN), the AA more specifically linked to ARG metabolism, correlated with AAIR better than ARG and, for any given AAIR, was lower after trauma than after the development of sepsis. Correlations of ORN with other AA levels were poorer than those found for ARG, however ORN was directly related to white blood cell and platelet count, fibrinogen, transferrin, cholesterol and many AA clearances.

These data show that changes in ARG in trauma and sepsis are correlated with changes in other AA and, within these correlations, reconfirm a tendency to lower ARG in trauma compared to sepsis. The strong correlation with lysine warrants a deeper assessment of the practical implications of interdependency between these two AA. The data also suggest that changes in plasma ORN in trauma and sepsis may reflect adequacy of AA substrate to support acute-phase and other synthetic processes.

The relationship between plasma albumin, other proteins and variables, and age in the acute phase response after hepatectomy in man

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A large series of plasma albumin (ALB) and additional blood and clinical measurements, prospectively performed on 92 liver resection patients (PTS), were processed to assess the correlations between ALB, other plasma proteins, additional variables and clinical events.

More than 500 measurements were collected. These were performed preoperatively and at postoperative day 1, 3 and 7 in all PTS, and subsequently only in those who developed complications or died. Each measurement included ALB, total protein (PROT), fibrinogen, total cholesterol (CHOL), triglycerides, glucose, urate, bilirubin, creatinine, urea nitrogen, iron binding capacity (IBC), sodium, chloride, potassium, calcium (CA), magnesium, phosphate, prothrombin activity (PA), partial thromboplastin time, aspartate and alanine aminotransferase, alkaline phosphatase (ALKPH), gamma-glutamyltranspeptidase, cholinesterase (CHE), hematocrit (HCT), hemoglobin, blood cell counts, lymphocyte count (LYM), other variables. Demographics, data on the operation, intraoperative liver ischemia, transfusions, fluid and colloid infusions, postoperative course and complications (mainly sepsis and/or liver insufficiency), presence of cirrhosis, neoplastic disease, previous chemotherapy, were also considered.

In PTS who recovered normally ALB was 4.3 ± 0.4 g/dL (mean $\pm$ SD) preoperatively, 3.7 ± 0.4 at day 1 and 3, and 3.9 ± 0.4 at day 7. In PTS with complications it decreased for a more prolonged period. In non-survivors it was 3.4 ± 0.4 preoperatively, 3.0 ± 0.4 at day 1, and then decreased further. Regression analysis showed, for postoperative ALB, direct correlations with CHE, CHOL, IBC, CA, PROT, PA, HCT, LYM (r^2 from to 0.53 to 0.18, $p < 0.001$ for all) and weaker correlations with other variables and exogenous ALB dose. Many of these correlations were present also preoperatively. ALB was related inversely to age (AGE) and to the occurrence of complications and death ($p < 0.001$ for all). The more relevant overall correlations were:

$$\mathrm{CHE} = 287(2.014)^{\mathrm{ALB}} \ [r^2 = 0.53];$$

$$\mathrm{CHOL} = 16.5(1.61)^{\mathrm{ALB}} \ (1.001)^{\mathrm{ALKPH}} \ [r^2 = 0.50]$$

$$\mathrm{PA} = 14 + 16(\mathrm{ALB}) \ [r^2 = 0.26]; \mathrm{IBC} = 2.5 + 0.005(\mathrm{ALB}) \ [r^2 = 0.42]$$

$$\mathrm{ALB} = 5 - 0.013(\mathrm{AGE}) - \{0.5 + 0.003(\mathrm{AGE})_{\mathbf{COMPL}} + 0.012(\mathrm{AGE})_{\mathbf{DEATH}}\}_{\mathbf{POSTOP}} \ [r^2 = 0.55]$$

($p < 0.001$ for each regression, and each coefficient in the regressions).

Continuous variables (units): CHE: U/L, nv > 4500; CHOL: mg/dL; ALKPH: U/L, nv < 279; PA: % of standard reference; IBC: mg/dL; AGE: years. Discontinuous variables (COMPL = patients with complications; DEATH = nonsurvivors; POSTOP = postoperative measurements) label the change in regression slope or intercept which is associated with the corresponding condition.

These results support the concept that pathophysiologic events, through various mechanisms, are the main driving factors setting ALB. The correlations with age and postoperative outcome support the concept that hypoalbuminemia is a marker of pathophysiologic frailty associated with increasing age, and amplified by the challenges of postoperative illness.

N-chlorotaurine and ammonium chloride: a highly effective antiseptic preparation

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Introduction: The bactericidal properties of the innate antiseptic N-chlorotaurine (NCT) are significantly enhanced in the presence of ammonium chloride which induces the formation of NH_2Cl whose prominent bactericidal properties are well known.

Methods: The reaction between NCT and ammonium chloride was monitored by UV-spectrophotometry, pH, and iodometric titration.

Results: N-chlorotaurine oxidized ammonium only to the stage of mono-chloramine, NH_2Cl, while with other active chlorine compounds like chloramine T (CAT), chloroisocyanuric acid derivatives, hypochlorites (NaOCl, $CaOCl_2$) the reaction proceeded forming also $NHCl_2$ and

NCl_3 which both are unpleasant smelling and therefore unwanted by-products.

Conclusions: The combination NCT and NH_4Cl turned out to be a highly effective antiseptic preparation with sufficient stability for an application in practice.

Influences of nutritional and genetic determinants of homocysteine in neurodegeneration: new evidences in methyl-deficient rats and in association studies with Alzheimer and Down syndrome

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Homocysteine metabolism is associated with the risk of sporadic Alzheimer's disease (SAD) and Down syndrome (DS), an Alzheimer type dementia with an accelerated IQ decline. These two Alzheimer type dementia involve complex multi-genetic and gene nutrient interactions. We evaluated the association of homocysteine with cognitive functions in a newborn rat model deficient in dietary methyl precursors. Homocysteine was increased in plasma and in specific area of the brain, including hypocampus and cerebellum. Behavior tests showed dysfunction of motricity coordination and of cognition. We evaluated also the association of homocysteine and related polymorphisms (*MTHFR*, *MTR*, *MTRR and TCN*) with dementia severity and cognitive decline in 180 SAD cases and 131 patients with trisomy 21. The patients were from a specialized center of Sicily and the matched controls were recruited in the same area. Homocysteine (upper tercile) was associated with SAD risk, with an odds ratio of 2.8 (95% confidence interval: 1.54–5.22, $P = 0.0008$), which was increased 2.2-fold by *MTHFR 677 T* (odds ratio: 6.28, 95% confidence interval: 2.88–16.20, $P < 0.0001$). *MTR 2756 AA* genotype was at risk of severe dementia (odds ratio = 2.97, 95% confidence interval: 1.23–7.21, $P = 0.016$). In DS patients, IQ was significantly lower in cases with t-Hcys $> 7.5\,\mu$mol/l (median) and in carriers of *MTHFR 677 T* allele and *MTHFR 677 T/TCN 776 G* combined alleles ($P = 0.0013$, $P = 0.0165$ and $P = 0.0074$, respectively). In multivariate analysis, t-Hcys was a significant determinant of IQ, independently of age ($P = 0.0037$). t-Hcys $> 7.5\,\mu$mol/l (median) was associated with the risk of severe/profound mental retardation (odds ratio: 3.0, 95% confidence interval: 1.4–6.5, $P = 0.0044$). *TCN1 776 G* allele was the single genetic trait associated to IQ decline, with a rate 1.5-fold greater in carriers than in non-carriers (-2.5 ± 1.3 vs. -1.6 ± 0.7, $P = 0.0119$). In conclusion, hyperhomocysteinemia is associated to cognitive and motricity dysfunctions in methyl-deficient newborn rats. Its influence on dementia severity and cognitive decline in SAD and/or DS is modulated by its related genetic determinants *MTHFR*, *MTR and TCN*.

Association of homocysteine with left ventricular dysfunction, independently from coronary artery disease

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Objective: Elevated plasma homocysteine is a risk factor for coronary artery disease (CAD) and for the development of acute congestive heart failure. However, its association of homocysteine with left ventricular systolic dysfunction according to the presence of coronary artery disease has not been specifically assessed.

Methods: A prospective study evaluated this relation by univariate and multivariate analyses in 560 consecutive patients referred for diagnostic coronary angiography, excluding those with recent myocardial infarction or unstable angina.

Results: The population consisted of 398 CAD patients and 162 patients without CAD on coronary angiography, with a median age of 63 years (inter-quartiles, 53 and 71) and an overall proportion of men of 76.8%. Homocysteine level was significantly higher in the 138 patients with left ventricular ejection fraction (LVEF)<40%, compared to those with a higher LVEF ($P < 0.0001$). It was significantly correlated with LVEF and NT-pro-BNP in univariate regression ($r = -0.234$, 95% CI: -0.31–-0.15, $P < 0.0001$ and $r = 0.306$, 95% CI: 0.18–0.42, $P < 0.0001$, respectively) and in a multiple regression, which considered potential confounders and risks factors for CAD and heart failure ($P < 0.0001$ and $P = 0.0073$, respectively). In stepwise logistic regression analysis, homocysteine $> 15\,\mu$mol/L was associated with LVEF $< 40\%$ in the whole population (odds ratio: 2.0, 95% CI: 1.3–3.1, $P = 0.0005$) and in patients without evidenced CAD (odds ratio: 3.6, 95% CI: 1.7–7.5, $P = 0.0005$).

Conclusion: Our results show an association of homocysteine levels with left ventricular systolic dysfunction, independently from that with coronary artery disease.

The influence of *β*-alanine supplementation and training on the muscle carnosine content in human *v. lateralis*, and the effect of this on exercise performance

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Carnosine (β-alanylhistidine) (Carn) occurs in high concentrations in muscle where it contributes to H^+ buffering (pKa of imidazole ring is 6.83). In contrast to other buffers (Pi & HCO_3) its concentration varies greatly between muscles from different species, and even between different fibre types, accounting for most of the variability in physicochemical buffering. Type II muscle fibres have 2–3 times the concentration of type I fibres (Harris et al., 1998). In humans its content ranges from $17.5 \pm 4.8\,\text{mmol} \cdot \text{kg}^{-1}$ dry muscle in females to $21.3 \pm 4.2\,\text{mmol} \cdot \text{kg}^{-1}$ dm in males (Mannion et al., 1992), with higher concentrations in athletes such as sprinters (Parkhouse et al., 1983) and lower concentrations in elderly, non-active subjects.

1) Supplementation: L-Carnosine (and its methyl derivative, anserine) ingested with meat or as a supplement are hydrolysed to histidine (His) and beta-alanine (β-Ala). Carn in muscle is therefore synthesized *in situ* and β-Ala availability appears to be rate limiting. There is a dose dependent increase in the plasma β-Ala concentration with 10–40 $\text{mg} \cdot \text{kg}^{-1}$ bwt β-Ala supplementation; loss *via* urine is minimal up to $40\,\text{mg} \cdot \text{kg}^{-1}$ bwt. However, subjects may show symptoms of flushing/paraesthesia above $10\,\text{mg} \cdot \text{kg}^{-1}$ bwt. Four weeks supplementation with **A**: $3.2\,\text{g} \cdot \text{d}^{-1}$ of β-Ala; **B**: 4.0 g increasing from week 1 to 4 to $6.4\,\text{g} \cdot \text{d}^{-1}$ of β-Ala; **C**: 10 g L-Carnosine increasing from week 1 to 4 to $16\,\text{g} \cdot \text{d}^{-1}$ (i.e. isomolar with respect to β-Ala in treatment **B**), increased the muscle (*v. lateralis*) Carn content by 42.1 ± 14.9, 64.2 ± 42.7 & $65.8 \pm 31.8\%$, respectively. There was no decrease in muscle taurine, which shares the same transporter as β-Ala. Recently we have extended supplementation with β-Ala, with (a) and without (b) co-supplementation with creatine monohydrate,

to 10 weeks. Muscle Carn content in this case was increased by: a) 80.1 ± 26.7 & b) 76.3 ± 16.6%, with increases in both types I and II fibres.

2) Training: Intense physical training is capable also of increasing muscle Carn. Body builders have muscle (*v. lateralis*) Carn contents 100% greater than untrained subjects. 12 days intense training of elite Korean speed skaters increased their muscle content by 87.0 ± 68.7%, the change being negatively correlated to the initial muscle content. Today we are investigating the combined effects of training and β-Ala supplementation.

3) Exercise performance: Suzuki et al. (2002) earlier claimed a positive relationship between performance in the latter half of 30-s maximal ergometer sprinting and the muscle Carn content. We have not observed any effect of Carn elevation at this exercise intensity but have observed an effect on work-done and endurance time when cycling to exhaustion at 110% 'power max' (predicted pre-supplementation endurance time: 2.5 min). A similar increase was seen with 5 days creatine (Cr) loading ($20\,g \cdot d^{-1}$) but effects were not additive when Cr and β-Ala were combined. This may reflect the limitations of the exercise model.

Conclusion: Muscle Carn is increased by supplementation and training. Elevation may affect exercise performance above the anaerobic threshold but less likely at supramaximal levels where pH change may not be limiting. As a dietary supplement, β-Ala needs to be seen in the same category as carbohydrate and Cr.

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The distribution of carnosine and taurine in different muscle fibre types from human *v. lateralis* and the effects of beta-alanine supplementation

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Previous studies have shown that in equine, camel and human muscle highest concentrations of carnosine (β-alanylhistidine) (Carn) are found in type II fibres. In equine the type II:I ratio may be as high as 5, but 1.5 to 2.5 in humans. In contrast taurine (Tau) shows the opposite distribution which can be so extreme in equine muscle that type II fibres may be $<2\,mmol \cdot kg^{-1}$ dry weight in contrast with 50–$70\,mmol \cdot kg^{-1}$ dwt in neighbouring type I fibres. With a pKa of 6.83 for the imidazole ring, Carn is a potent intracellular pH 7 buffer and important to H^+ buffering during exercise above the anaerobic threshold. The function of Tau is less clear but quantitatively mirrors Carn and may be involved in osmoregulation. Carn synthesis in muscle is limited by β-Alanine (β-Ala) availability from *in-vivo* synthesis and the diet, and we have recently shown that Carn can be increased 80% with 10 wks β-Ala supplementation. High concentrations of β-Ala, however, may interfere with Tau absorption and in high doses in animal models has been used to cause Tau depletion in cardiac and skeletal muscle. From 6 males supplemented with β-Ala for 10 wks, 30–40 single muscle fibres were dissected from Pre and Post biopsies of *v. lateralis*; 3 of these subjects additionally received $2 \times 7d \times 20\,g \cdot d^{-1}$ creatine monohydrate (CrM). From each fibre a small piece was cut, solubilised and subjected to SDS-PAGE gel electrophoresis with silver staining to identify MHC isoform patterns (I, I/IIa, IIa, IIa/IIx). The remaining portion of each fibre was weighed on a quartz-fibre fishpole balance, extracted with ice-cold water and 10+ type I and IIa fibres, and any IIx, I/IIa or IIa/IIx hybrids were assayed for Carn and Tau by fluorescence-HPLC as OPA derivitates. Mean (SE) Carn & Tau contents before and after supplementation were 21.3 (2.6) & 37.3 (2.7) and 42.3 (2.6) & 36.6 (4.3) $mmol \cdot kg^{-1}$ dwt. CrM supplementation did not affect Tau or the increase in Carn with β-Ala supplementation. Single fibre Carn contents were normally distributed about the mean but 1.3 to 2.5× greater in IIa compared to type I fibres. Tau showed the reverse distribution. Carn increased in both fibre types after β-Ala supplementation but Tau was unchanged.

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Changes in protein synthesis in skeletal muscle, liver and gut after glutamine and/or branched-chain amino acid infusion

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Glutamine (Gln) and branched-chain amino acids (BCAAs; valine, leucine, and isoleucine) are considered as an effective nutrient in treatment of severe illness. Considering that BCAAs are an important precursor of Gln synthesis in skeletal muscle and that Gln administration significantly decreases leucine oxidation (JPEN 24: 215–22, 2000), it may be suggested, that simultaneous administration of Gln and BCAAs affects protein metabolism more significantly and/or by a different way than theirs separate administration.

Male Wistar rats weighing 220–250 g were infused for 6 hours by 5 ml of 1.75% Gln solution (n = 7), 1.75% BCAA solution (n = 7), 1.75% Gln + BCAA solution (n = 7) or saline (Control, n = 7). Changes in protein synthesis in gastrocnemius muscle, liver and small intestine were evaluated using ''constant infusion'' of L-[1–14C]leucine and by a ''flooding method'' with [4,5-3H]phenylalanine. Amino acid concentrations were measured using HPLC. Statistical analysis was performed using ANOVA and Bonferroni test. $P < 0.05$ was considered significant.

The obtained data showed a significant increase in protein synthesis in skeletal muscle in Gln and Gln + VLI infused rats. The significant increase in protein synthesis in small intestine was observed only in Gln + VLI infused animals. Changes in protein synthesis in the liver were insignificant. In Gln infused animals a significant increase in Gln and a decrease in BCAA concentrations were observed in blood plasma. However, a significant increase in BCAA levels was observed in several tissues of Gln, BCAA and Gln + BCAA infused animals.

We conclude that simultaneous administration of Gln and BCAA may favourably affect protein synthesis in several tissues, particularly in small intestine, and that Gln infusion has a marked effect on BCAA concentrations in body fluids.

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Allyl disulfide as donor and cyanide as acceptor of sulfane sulfur in the mouse tissues

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Cytoplasm of mammalian glial cells was reported to contain Gomori-positive cytoplasmic granulation (GPCG), whose biological role is unknown. The present study attempted to discover conditions facilitating GPCG formation and to elucidate their relationship with sulfane sulfur metabolism. To address these problems, we investigated *in vivo* the effect

of allyl disulfide (DADS) and cyanide on the number of GPCG-containing glial cells in the mouse brain.

In parallel, sulfane sulfur level and activity of rhodanese and 3-mercaptopyruvate sulfur transferase (MpST) were determined in the mouse brain and liver.

Cyanide caused a drop in GPCG number in the brain, while activity of sulfur transferases and sulfane sulfur level remained unchanged. Slight but significant cyanide-induced rise in MpST activity was observed only in the liver, which indicates a possibility of enhancement of its detoxification in reaction with mercapropyruvate in this organ.

DADS, a sulfur donor, increased GPCG number in the brain, whereas activity of sulfur transferases and sulfane sulfur level did not change. However, in the liver, DADS elevated both sulfur transferase activity and sulfane sulfur level.

These observations suggest that DADS can constitute a source of sulfane sulfur for the liver, thereby activating anaerobic sulfur metabolism and sulfane sulfur transfer. Consequently, this leads to the increase in sulfane sulfur level in plasma, in which it is transported in the form of albumin hydropersulfides and can be used for cyanide detoxification. Therefore, it is not excluded that GPCG observed in the brain of mice and other mammals can be a source and a store of sulfane sulfur in mammals.

The effect of α-melanocyte stimulating hormone on ethanol-induced gastric damage in the rat

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The pro-opiomelanocortin-derived tridecapeptide α-melanocyte stimulating hormone (α-MSH) exerts broad anti-inflammatory actions in various experimental models due to its ability to limit induction of genes encoding pro-inflammatory cytokines, cell adhesion molecules and the inducible nitric oxide synthase.

Aim: To investigate the effect of α-MSH on ethanol-induced gastric ulcer in rats and to evaluate the mechanisms of action.

Materials and methods: Sprague-Dawley rats of both sexes (200–250 g; $n = 7–8$/group) received 1 ml 75% ethanol or saline orally. α-MSH was given (25 μg/rat; ip) alone or following COX-1 inhibitor FR122047 (10 mg/kg; po), COX-2 inhibitor NS-398 (5 mg/kg; ip), CGRP antagonist $CGRP_{8-37}$ (100 μg/kg; ip), somatostatin antagonist (10 μM/kg; ip), sulfhydryl blocker NEM (10 mg/kg; ip) or atropin (1 mg/kg; ip). Gastric mucosa was scored macroscopically and microscopically following decapitation 30 min after ethanol. Malondialdehyde (MDA; product of lipid peroxidation), antioxidant glutathione (GSH) and myeloperoxidase activity (MPO; indicator of neutrophil infiltration) and mast cell counts were assessed in tissue samples. Data were analysed with ANOVA followed by Tukey-Kramer test.

Results: Ethanol induced gastric hemorrhagic lesions characterized by increase in MDA, MPO activity and mast cell count and decrease in GSH compared to control. α-MSH decreased the extent of tissue injury ($p < 0.05–0.001$) and reversed MDA and MPO levels ($p < 0.01–0.001$) and mast cell count ($p < 0.01$). The effect of α-MSH on lesions was reversed by all agents ($p < 0.05$) except atropin. The effect of α-MSH on MDA was reversed by atropin ($p < 0.001$), somatostatin antagonist ($p < 0.001$), $CGRP_{8-37}$ ($p < 0.05$) and NEM ($p < 0.001$). The effect of α-MSH on MPO was reversed by somatostatin antagonist ($p < 0.05$) and COX-2 inhibitor ($p < 0.05$).

Conclusion: α-MSH is beneficial in a rat model of gastric ulcer *via* mechanisms involving mainly somatostatin, sulfhydryls and CGRP and partially COX and muscarinic receptors of the cholinergic pathway.

Branched-chain amino acids promote the gene expression of taurine biosynthetic enzyme, cysteine dioxygenase

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Alterations in hepatic metabolism of sulfur-containing amino acids are often found in cirrhosis and supposed to be related to its complications. There are many reports referred to abnormal expressions of the genes involved in methionine and cysteine metabolism. In this study we focused on hepatic cysteine dioxygenase (CDO), a critical regulator of metabolism of cysteine to sulfate, taurine and glutathione.

In CCl_4-induced cirrhotic rats, we found that hepatic enzymatic activity and the gene expression of CDO were significantly reduced. In order to clarify the molecular basis of this suppression, we further explored the regulation of its gene expression using human hepatoma cell line (HepG2). First of all, we found that CDO gene expression was down-regulated by transforming growth factor-β (TGF-β), one of the typical inflammatory cytokines involved in pathogenesis of cirrhosis. Inhibitor experiments revealed that the suppression by TGF-β was attributed to transcriptional control, which was mediated by MEK/ERK pathway.

Furthermore, we investigated the effect of branched-chain amino acids (BCAA) on CDO gene regulation in HepG2, since BCAA would be well known to have some beneficial effects on cirrhosis, such as improvement of nutritional status and ameliorating hypoalubuminemia in the decompensated cirrhotic patients. We found that BCAA, especially leucine, promoted CDO gene expression at transcriptional level in a mTOR-independent manner. Moreover, BCAA could also promote the CDO gene expression even when it is down-regulated by TGF-β.

In summary, the present study indicated that the gene expression of CDO, a rate-limiting enzyme of cysteine metabolism, was transcriptionally regulated by inflammatory cytokine and other amino acids, BCAA, which was not supposed to be involved in metabolic crosstalk with cysteine. Our results suggest that BCAA may be therapeutically useful to improve the abnormalities in hepatic cysteine metabolism often found in cirrhosis.

Comparison of taurine chloramine (Tau-Cl) and taurine bromamine (Tau-Br) effects on rheumatoid arthritis synoviocytes

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Fibroblast-like synoviocytes (FLS) participate in rheumatoid arthritis (RA) chronic synovitis by producing pro-inflammatory cytokines (IL-6, IL-8), growth factors (VEGF) and other inflammatory mediators (PGE_2, NO). We have previously reported that Tau-Cl, generated by neutrophils in a reaction of HOCl with taurine, inhibits *in vitro* some of these pathogenic RA FLS functions. However, taurine also reacts with HOBr originated from eosinophils, resulting in generation of Tau-Br, the compound of poorly known activities. Therefore, we investigated the effect of Tau-Br on RA FLS functions and compared it to Tau-Cl anti-inflammatory activities. We observed that RA FLS were more sensitive to Tau-Br ($> 300\,\mu$M) than Tau-Cl ($> 500\,\mu$M) cytotoxicity. When applied at noncytotoxic concentrations: (i) Tau-Cl and Tau-Br inhibited IL-6 and PGE_2 production with similar potency ($IC_{50} \approx 250\,\mu$M), (ii) only Tau-Cl inhibited VEGF and IL-8 synthesis ($IC_{50} \approx 400\,\mu$M), while (iii) none of these compounds affected NO generation and iNOS expression.

Moreover, Tau-Cl selectively downregulated COX-2, while Tau-Br had no effect on either COX-1 or COX-2 protein expression, suggesting that the latter compound acts probably by either inhibiting enzymatic activity of COX or by promoting PGE_2 degradation. In conclusion, we report that Tau-Cl is more effective than Tau-Br in normalization of pro-inflammatory RA FLS functions. The differences in the effectiveness of tested compounds result from stronger cytotoxicity of Tau-Br toward these cells.

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Development of novel stress and infection protectors based on synthetic peptides

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The corticotropin-like peptides immunocortin (VKKPGSSVKV) and leucorticotropin (GKVLKKRR) were synthesized and their influence on the non-specific resistance of laboratory animals subjected to various stress factors was studied. It was found that both peptides, being injected intranasally at doses of 20–40 μg/kg, normalized the state of the corticosteroid-catecholamine system in adrenals and the histamine-diaminoxydase system in the myocardium of rats after acute hemorrhagic or hypothermic shock *in vivo*.

The immunostimulating beta-endorphin-like peptides immunorphin (SLTCLVKGFY), pentarphin (VKGFY), [Pro^3]pentarphin (VKPFY) and cyclopentarphin (cycloVKGFY) were synthesized. We found that these peptides at 1 nM concentration stimulate the anti-bacterial activity of mouse peritoneal macrophages towards *Salmonella typhimurium* virulent strain 415 *in vitro*. Pentarphin administered intraperitoneally at a dose of 20 μg/mouse on day 7, 3 and 1 prior to the isolation of cells enhanced the activity of peritoneal macrophages as well as T and B lymphocytes.

Analysis of human urine for homocysteine-thiolactone by capillary electrophoresis

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One of the possible mechanisms explaining homocysteine toxicity in humans leads *via* its highly reactive metabolite homocysteine-thiolactone. This thioester has ability to modify proteins by forming linkages in which homocysteine forms amide bond with ε-amino group of protein lysine side-chain. Modification with homocysteine-thiolactone decreases the normal physiological activity of proteins, affects protein structure, and has toxic effects on cells. It was shown that human body eliminates homocysteine-thiolactone by urinary excretion. The data regarding the levels of this important metabolite in the human body are limited, chiefly due to a shortage of simple and reliable analytical methods. A few methods available so far use high-performance liquid chromatography or gas chromatography/mass spectrometry. In this presentation we described an attempt of application of capillary electrophoresis, known for its high resolution, selectivity, speed, and minute sample requirements, for determining homocysteine-thiolactone in urine. Capillary zone electrophoresis mode separations were performed with the use of the Hewlett Packard HP^{3D}CE System equipped with UV-Vis diode-array detector and 52 cm $\times$ 50 μm I.D. bare fused-silica capillary. A 100 μl homocysteine-thiolactone containing urine was mixed with 200 μl of acetonitrile and an aliquot was injected (48 $\times$ 35 mbar $\times$ s) into the system. The background electrolyte was 0.20 M pH 7.46 TRIS/HCl buffer, the temperature 25°C, and the voltage 20 kV. The detector response, at analytical wavelength 240 nm, was linear ($R^2 = 0.9995$) over a calibration range 10–150 nmol/ml urine. The assay imprecision expressed as relative standard deviation was within 4.1 and 2.6% for the bottom and top of the calibration range, respectively. The lower limit of quantitation was 10 nmol/ml; 500 fmol on capillary.

Taurine protects against the neurotoxic effects of guanidino compounds

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Chronic renal failure (uraemia) is accompanied by a wide range of CNS disturbances (uraemic encephalopathy) and increases in the plasma levels of numerous potential neurotoxins. Among the best characterised of these are guanidino compounds. The present study examines the effects of some guanidino compounds on cell volumes in incubated rat cerebrocortical minislices, it being predicated that swelling is *prima facie* evidence of cellular dysfunction. Significant swelling was observed in the presence of guanidine (3 μM), guanidinosuccinic acid (40 μM), methylguanidine (7 μM) and *N*-acetyl-L-arginine (1.5 μM). Swelling was decreased in a concentration-dependent manner by taurine, being abolished at 20 mM. Swelling was also abolished by the anto-oxidants ascorbic acid (400 μM) and butylated hydroxytoluene (500 μM), by the free redical scavenger *N*-acetyl-L-cysteine (10 mM), and by the lipid peroxidase inhibitor desmethyl tirilazad (100 μM). The ameliorative effect of 20 mM taurine was reduced by ~50% by the taurine transport inhibitor GES (1 mM) and by the $GABA_A$ receptor antagonist bicucculine (100 μM). The effects of GES and bicucculine were additive. It is hypothesized that taurine exerts it protective affect against guanidino-dependent swelling in 2 ways. One (intracellular) is transport-dependent and involves suppression ROS formation, probably involving inhibition of lipid peroxidation. The other (extracellular) involves $GABA_A$ receptor activation with consequent cellular hyperpolarization. This has the effect of maintaining the voltage-dependent Mg^{2+} block on the guanidino-sensitive NMDA receptor, thus preventing massive cellular Ca^{2+} entry and its cytotoxic consequences.

Searching for gelsolin amyloid formation

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Mutated gelsolin amyloidogenic fragments 173–243 (G173–243) and 173–202 (G173–202) cause Finnish familial amyloidosis disease (FAF). The mechanism of amyloidogenesis is not clear. This work using molecular dynamics (MD) investigates interactions of gelsolin amyloidogenic fragments G173–243 and G173–202 after cleavage from the x-ray structure of gelsolin (1DON), and interaction of G173–202 with dimyristoylphosphatidylcholine (DMPC) bilayers.

Fragment G173–243 immersed in a periodic water box, minimized, subjected to MD simulation, using the AMBER 5.0 force field, and

the NTP (constant number of molecules, temperature, pressure) protocol, T = 298 K, partly lost its(α-helical structure after 1154 ps of the MD run suggesting that G173–243 could unfold before or during amyloidogenesis.

Two possible amyloid strand structures built from G173–202 units, using twelve G173–202 for initial side-to-side orientation (12StS) and eight G173–202 for initial face-to-face orientation (8FtF), were minimized, subjected to MD at T = 313 K, NTP. With the 12StS after 1100 ps the G173–202 fragments initially placed in one plane tended to associate by forming intermolecular hydrogen bonds between the strands, the strands being at an angle of 10–20 degrees per subunit. For the 8FtF initially the component β-sheets were in the stacking orientation. During the course of simulation pairs of them turned side to side forming intermolecular backbone hydrogen bonds, molecules approached closer, and the dimension of the system decreased, showing the tendency of the G173–202 fragment to amyloid-fibril assembly.

Fragment G173–202 was put in two different orientations 9 Å away from a DMPC bilayer and subjected to MD at T = 312 K, NTP. After 100 ps of MD, the G173–202 fragment in either initial orientation adhered to the DMPC membrane, retaining β-sheet structure, suggesting that a lipid membrane could bind G173–202 fragments and serve as a germ for amyloidogenesis.

Afterwards one more amyloidogenic fragment G173-202 was placed in the vicinity of G173–202 adhered to the DMPC membrane of the previous system "start-2", and the new system was subjected to NTP MD simulations at T = 312 K for 3744 ps. After 700 ps of MD, the newly added amyloidogenic fragment G173–202 was interacting with the membrane attached fragment G173–202 and after 1465 ps they were bound with hydrogen bounds, indicating the possible mechanism of membrane initiated amyloidosis and showing that fragments G173–202 can interact forming β-sheet.

Subsequently all systems were placed in a periodic lipid-water box and subjected to MD simulations to investigate of the stability of the structures.

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3-Ethylcystathionine, a novel amino acid formed during the catabolism of 3-hydroxynorvaline in pregnant female mice

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Several toxicological studies have previously been executed on 3-hydroxynorvaline (HNV), a microbial L-threonine analogue. HNV proved to be able to inhibit DNA synthesis and protein glycosylation in fibroblasts and/or micro-organisms. We recently demonstrated that HNV was embryotoxic (growth retardation) and teratogenic (neural tube defects) to chicken- and mouse embryos. The metabolic fate of HNV in higher animals is, as yet, unknown. The catabolic breakdown of HNV in pregnant female mice was subsequently investigated. Pregnant Han.NMRI females were dosed with HNV (gavage) on days 7, 8 and 9 of gestation. Controls received saline. Urinary organic acid, acylcarnitine and amino acid profiles of control and HNV-treated females were assessed (24-h urine samples). GC-MS studies revealed that selected optical isomers, in a racemic mixture of HNV isomers, were rapidly metabolized. 2,3-Dihydroxypentanoic acid appeared to be the main metabolic intermediate. HNV caused numerous metabolic perturbations in experimental animals. HNV metabolites seemingly inhibited β-oxidation, isoleucine catabolism and ketone body utilization, most probably by inhibiting the activity of keto-thiolase enzymes, involved in these metabolic pathways. A metabolic profile, exhibiting similarities to a 2-methylacetoacetyl-CoA thiolase deficiency, appeared to have been induced. HNV also proved to be a substrate for cystathionine-β-synthase, producing 3-ethylcystathionine, a novel amino acid. The latter compound appeared to have been rapidly catabolized to α-ketobutyric acid and 3-ethylcysteine. The presence of 3-ethylcysteine was confirmed in urine of HNV-treated mice by means of GC-MS. We conclude that HNV is rapidly catabolized in the mouse, resulting in numerous metabolic perturbations.

Immunochemical characterization of altered allergenic contents of manufactured food due to varying processing conditions and different food matrices

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Food processing may have pronounced impacts on the protein contents of manufactured products with respect to structural and hence immunological changes. Given the complexity of commercial food matrices molecular mechanisms occurring during food processing are affected by several complementary parameters. The determination of allergens faces the problem of their altered accessibility on the protein level and/or changes in their epitopes dependent on the extent of denaturation evoked by a certain technological treatment. Fermentation of dairy products, which constitutes one possibility to reduce allergenicity, was investigated by immunochemical methods (ELISA, Western Blot) and peptic digestion of the major whey allergen β-lactoglobulin. The fermentation of milk by different microorganisms can reduce the resistance of native β-lactoglobulin against peptic digestion. Increased proteolytic instability of the allergen highly indicates a reduced risk for allergic patients. Various acidified and fermented milk products were screened for altered affinity properties of anti-β-lactoglobulin antibodies to the content of allergens in correlation with the metabolism of different fermentation bacteria cultures and aging of these fermentation products.

The role of heme oxygenase −1 (HO-1) in anti-inflammatory properties of taurine chloramine and taurine bromamine

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The myeloperoxidase – halide system of neutrophils generates hypohalous acids (HOCl, HOBr) *in vivo*. Both can react with beta amino-acids to produce less toxic haloamines. As taurine is the most abundant free amino acid in leukocyte cytosol, the major haloamines are taurine chloramine (TauCl) and taurine bromamine (TauBr). TauCl: (i) exerts antibacterial activity; (ii) down-regulates the production of inflammatory mediators by cells engaged in inflammatory response; (iii) induces apoptosis in some immune cells; (iv) enhances protein immunogenecity by chlorination. Much less is known about biological properties of TauBr.

Heme oxygenase −1 (HO-1), similarly to taurine, has been implicated in the cytoprotective defense response against oxidative injury. HO-1 is an inducible enzyme that degrades heme to biliverdin, free iron and carbon monoxide. It was shown that CO may inhibit the production of pro-inflammatory cytokines in macrophages. Moreover, induction of

HO-1 by several compounds has been claimed to decrease activation of NFkB signalling and to inhibit macrophage NOS-2 induction. Thus, there are striking similarities between influence of TauCl and HO-1 inducers on LPS-dependent NOS-2 induction in macrophages.

The aim of our studies was to determine whether HO-1 mediates the anti-inflammatory effect of TauCl and TauBr in macrophages.

TauCl and TauBr, in a similar, dose-dependent manner, inhibited the expression of NOS-2 and the production of TNF-α, IL-10, IL-12p40 in activated macrophages. Surprisingly, TauCl and TauBr induced expression of HO-1 in both non-activated and LPS-activated macrophages. Importantly, an inhibitor of HO-1 activity, chromium III mesoporphyrin, affected the tested immunoregulatory activities of TauBr and TauCl. These results suggest that at a site of inflammation, haloamines may provide a link between taurine-dependent and HO-1-dependent response to oxidative stress.

Advances in the quantitation by HPLC of the o-phthalaldehyde derivatized amino acids and amines

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This paper is intended to be a critical evaluation of all those proposals that have been published for the analysis of amino acids (AAs) and amines (As) derivatized by *o*-phthalaldehyde (OPA) in the presence of various SH-additives (2-mercaptoethanol, 3-mercaptopropionic acid, N-acetyl-L-cysteine, ethanethiol).

On the basis of literature data and own experiences [1–10] derivatization/quantitation issues were investigated both from analytical point of view and in respect of the reaction-mechanism of the process. Stoichiometric and HPLC/MS/API(ESI) fragmentation studies of derivatives leaded to the explanation of the exceptional instability of such very important derivatives as glycine, GABA, β-alanine, histidine, ornithine, lysine, all aliphatic mono/diamines.

Effects of N_Ω-nitro-L-arginine-methylester-hydrochloride (L-NAME) on neutrophil (PMN) free amino acid and α-keto acid profiles and immune functions *in vitro*

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The objective of this study was to determine the dose as well as duration of exposure-dependent effects of L-NAME, an inhibitor of nitric oxide synthase (•NOS), on polymorphonuclear neutrophil (PMN) free amino and α-keto acid profiles and, in a parallel study, on PMN immune functions. Following L-NAME (1 mM), intracellular amino and α-keto acid pools remained unaffected. Concerning PMN immune function markers, L-NAME significantly increased both superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) formation, while myeloperoxidase activity remained unaltered. We therefore believe that inhibition of •NOS in PMN is not connected with significant changes or malfunctions of major biochemical pathways. This is not surprising taking into consideration that •NO is not exclusively generated by the reaction of L-arginine and •NOS. Indeed, there is also evidence for a novel nonenzymatic pathway for the generation of •NO by the reaction of H_2O_2 and D- or L-arginine or the involvement of glutamine in •NO-production by immunostimulated neutrophils. Moreover, regarding PMN immune functions we suggest that decreases in intra- and extracellular •NO, for example induced by inhibition of •NOS, "activates" O_2^-- and H_2O_2-generation as evidenced by an inverse correlation between PMN arginine derived •NO-generation and O_2^--formation (the direct toxcity of •NO is modest, but is greatly enhanced by reacting with O_2^- to form peroxynitrite). Regarding our results we therefore believe that increased O_2^-- and H_2O_2-formation may be due to either 1) a loss of •NO- und O_2^--mediated peroxynitrite connected with an increased H_2O_2-production from O_2^- or 2) due to an increased superoxide dismutase (SOD) activity or SOD-mimics, or both.

Effects of S-nitroso-N-acetyl-penicillamin (SNAP) on neutrophil (PMN) free amino acid and α-keto acid profiles and immune functions *in vitro*

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We have examined the dose as well as duration of exposure-dependent effects of SNAP [nitric oxide (•NO) donor] on polymorphonuclear neutrophil (PMN) free amino- and α-keto acid profiles and, in a parallel study, on PMN immune functions. In the presence of 100 μM SNAP (≈5.6 μM •NO/min) significant decreases in PMN gln, glu, asn, asp, arg, orn, cit, h-tau, ala, ser, gly, thr, α-KG, PYR, α-KB, α-KIV, α-KIC, PhePyr, α-KMV, myeloperoxidase activity, superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) formation were observed. With regards to our results we suggest that changes in PMN amino and α-keto acids (for example induced by exogenous •NO), particularly, may be the result of relevant inhibition or modulation of major intracellular biochemical pathways or amino and α-keto acid transport. Moreover, we believe that imbalances in PMN amino- and α-keto acid substrate pools, not only altered in concentration, but also of unsuitable composition may have an unintentional influence on PMN immune functionality. Regarding PMN immune function markers, SNAP induced an undisputed reduction in PMN oxygen-dependent cytotoxic responses and inflammatory enzyme activity. This is not surprising, because considerably impaired amino as well as α-keto acid metabolism (deficiencies or excesses) can certainly result in limitations of important PMN functions, despite all other factors remaining equal. We therefore hypothesize that decreased PMN immune functions may be due to either 1) a loss of PMN-mediated reactive oxygen species (ROS) by sequestration of O_2^- by •NO to form peroxynitrite (connected with an decreased H_2O_2-production) or 2) undisputed inhibition of neutrophil function, or both.

N-Chlorotaurine as an endogenous antimicrobial agent in human medicine: results of clinical studies

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Introduction: N-chlorotaurine (NCT) is a long-lived oxidant produced by human leukocytes with broad-spectrum antimicrobial activity. It can

also be synthetized chemically as crystalline sodium salt. We performed three clinical phase II studies to evaluate efficacy and tolerability of this new antiseptic in otitis externa, epidemic keratoconjunctivitis, and chronic leg ulcers with a purulent coating.

Methods: Aqueous 1% NCT (55 mM) solutions were applied topically for 5 to 7 days. All studies were designed with a randomized test (NCT) and control group (standard medication). The otitis externa and epidemic keratoconjunctivitis study were double-blind. Evaluation was done clinically using a scoring system for subjective and objective symptoms and microbiologically.

Results: NCT was well tolerated in all indications, little burning in the eye for a few minutes in few patients like sweat and significantly less burning ($P < 0.05$) than by chloramine T in purulent crural ulcers were the only side effects. Granulation and re-epithelialization in ulcers appeared earlier in the NCT group ($P < 0.05$). NCT was equally effective in removal of the purulent coating than the stronger antiseptic chloramine T. The clinical symptoms of external otitis decreased 2 days earlier in the test group than in the control group treated with a combination of polymyxin B, neomycin, and hydrocortisone ($P < 0.01$). Regarding epidemic conjunctivitis, severe courses caused mainly by adenovirus type 8 showed a reduction of symptoms after 3 to 5 days, which was earlier than in the control group mock treated with gentamicin ($P < 0.05$).

Conclusion: The mild antiseptic NCT showed no considerable toxic or allergic side effects and is obviously effective in infections of different body regions.

Acknowledgements

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Diagnostic values of SELDI-TOF MS technology in alcoholic subjects

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Since personal and verbal reporting of alcohol use is not necessarily accurate, objective markers to assess alcohol consumption are required. The currently available markers, however, are limited in sensitivity and specificity for screening excessive drinkers. Therefore, searches for novel markers are warranted.

Recently, surface-enhanced laser/desorption ionization time-of-flight mass spectrometry (SELDI-TOF MS) has been successfully used to detect disease-associated proteins in complex biological specimens. We used the ProteinChip SELDI technology to generate comparative protein profiles of the consecutive serum samples obtained during abstinence from a total of 16 chronic alcoholic patients hospitalized for a rehabilitation program. An aliquot of the stored sera was used for the SELDI-TOF MS analysis with an anionic exchanger (SAX2) and a cationic exchanger (WCX2). We recognized two peaks (5.9 kD and 28 kD) the expression level of which remarkably changed during abstinence. These two proteins were partially purified and were subjected to amino acid sequencing. The 28 kD protein was identified as apoprotein A1 and the 5.9 kD was found to be a novel fragment of a known plasma protein. Notably, the relative intensities of the 5.9 kD peak down regulated in heavy drinkers significantly increased during abstinence even in non-responders of conventional markers for excessive drinking such as □-glutamyltransferase (GGT) and carbohydrate-deficient transferrine(CDT). In conclusion, the 5.9 kD peptide, complementary to GGT and CDT, is promising novel biomarker for alcoholic subjects.

Gas chromatographic mass spetrometric detection of free amino acid enantiomers in higher vertebrates

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Amino acid (AA) enantiomers were determined in brains, body fluids (urine, blood coagulate, serum, plasma), and faeces of animals belonging to nine out of eleven established taxonomic orders of vertebrates (Artiodactyla, Aves, Carnivora, Lagomorpha, Marsupalia, Osteichthyes, Primates, Rodentia, Tubilidentata). Free AA were isolated by means of cation exchangers and converted into volatile *N(O)*-perfluoroacylamino acid propyl esters. Derivatives of amino acids were separated into D- and L-enantiomers using enantioselective Chirasil®-L-Val capillary columns and detected by selected ion monitoring mass spectrometry. Quantification of amino acids was achieved by comparison of analytes with amino acid standards using L-norleucine as internal standard. Large relative amounts of D-Serine were determined in brains of all mammals, approaching 24% D-Ser in cerebrum of bovine. No D-Ser was detected in brains of birds. In body fluids the D-enantiomers of most proteinogenic L-amino acids were detected, largest absolute and relative amounts were found in urine. Therein, in many instances, quantities of D-Ala and D-Ser exceeded 50% relative to the respective L-enantiomers. Feeding of animals with diet fortified with DL-Met resulted in excretion of almost racemic Met in urine. D-amino acids were also abundant in faeces of animals. The data confirm that D-amino acids are common in body fluids and tissues of vertebrates.

Radiolabeling of human apolipoproteins using SH-reactive ^{18}F-labeling agents: a potential approach for characterization and differentiation of metabolism of native and modified lipoproteins by small animal positron emission tomography (PET) *in vivo*

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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 a new methodology for labeling of apolipoprotein B-100, the structural protein of both native LDL (nLDL) and oxLDL, with the positron emitter fluorine-18 (^{18}F; $t_{1/2} = 109.7$ min) by conjugation with SH-reactive *N*-[6-(4-[^{18}F]fluorobenzylidene)-aminooxyhexyl] maleimide ([^{18}F]MHAA) and the use of thusly ^{18}F-labeled LDL particles in dynamic PET studies in male Kyoto-Wistar rats. For labeling experiments, pools of chemically and biochemically well characterized human nLDL and oxLDL (modified by hemin/H_2O_2) particles, respectively, were used. Radiosynthesis of [^{18}F]MHAA started with the preparation of [^{18}F]fluorobenzaldehyde. In a one-pot procedure *N*-(6-aminooxyhexyl)maleimide was added to the generated aldehyde and after-wards [^{18}F]MHAA was isolated by HPLC. Preparation of [^{18}F]MHAA was achieved within 70 min with radiochemical yields of $34 \pm 5\%$ (corrected for decay) and purity of >96%. [^{18}F]MHAA was shown to react efficiently and selectively with SH-groups of various peptides and proteins under mild conditions. LDL labeling with [^{18}F]MHAA resulted in radiochemical yields of $20 \pm 10\%$ (corrected for decay) and specific radioactivity of 150–300 GBq/μmol. Radiolabeling of nLDL and oxLDL using [^{18}F]MHAA caused neither

additional oxidative structural modifications of LDL lipids and proteins nor alteration of their biological activity and functionality *in vitro*, respectively. The method was evaluated with respect to uptake of ^{18}F-labeled LDL in various human cell lines. Biodistribution studies in rats revealed high *in vivo* stability for the ^{18}F-labeled LDL. The metabolic fate of the ^{18}F-labeled LDL *in vivo* was delineated by dynamic PET studies using a dedicated small animal tomograph (spatial resolution of <2 mm). Data were compared to former studies using the NH_2-reactive ^{18}F-labeling agent *N*-succinimidyl-4-[^{18}F]fluorobenzoate. In conclusion, ^{18}F-labeling of LDL and the use of small animal PET provide a valuable tool for mapping sites of both nLDL and oxLDL metabolism in animal models *in vivo*.

A modeling approach to the study of arginine metabolism

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Mathematical modeling for simulation-based analysis is becoming a key tool for the development of systems biology, the analysis of the relationship among the elements in a system in response to genetic or environmental perturbations, with the goal of understanding the system as a whole. Recently, several interesting and relevant mathematical models of well known metabolic pathways have been published, including one describing the branched chain amino acid biosynthetic pathways of *E. coli*. We are interested in the modeling of arginine metabolism in mammals. The cationic amino acid arginine is the precursor of different biomolecules with very relevant physiological roles, such as urea, polyamines and nitric oxide, among others. Within the framework of arginine metabolism, we focus our interest in the branched pathway leading to either nitric oxide (*via* nitric oxide synthase) or polyamines (*via* arginase and ornithine decarboxylase). Although the different elements of this system are reasonably well known, our aim is to integrate the available data to explain its dynamics in a formal and predictable way. To fulfil this goal, we make use of a modeling and simulation approach that will be described in the present communication.

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Consequences of N-acylation strategies on 3D structure, binding and cytolytic properties of antimicrobial peptides

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Peptide-based lytic compounds represent a promising class of novel antimicrobial agents due to their simple molecular formula and their ability to escape microbial mechanisms for resistance. Acyl conjugation is known to significantly enhance certain antimicrobial properties but the molecular basis is ill-understood. We monitored the acylation effect through gradual increase of the acyl moiety chain length and modulated their hydrophobicity by acyl conversion to aminoacyl. Since the interaction with plasma membrane is often cited as an important step, eventually representing the ultimate site of action, the binding properties to bilayer models mimicking bacterial plasma membrane were investigated by Isothermal Titration Calorimetry and Surface Plasmon Resonance. Solution structures were investigated by Circular Dichroism and/or Nuclear Magnetic Resonance. The effects on cytolytic properties were investigated in culture media using bacteria, protozoa and human erythrocytes.

The combined data present compelling evidence in support of the hypothesis that N-acylation affects antimicrobial properties by modifying the peptide's electrostatic potential distribution in a manner that increases the potential area of contact, which consequently, facilitate binding properties to accessible plasma membranes. The data also suggest an approach to enhance potency of acylated short antimicrobial peptides by preventing hydrophobic interactions that lead to self-assembly in solution and thus to inefficacy against cell wall containing target cells.

Nitric oxide and nitrogen oxides synthesis by the mitochondrial nitric oxide synthase in rat liver

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Although it is know that mitochondrial nitric oxide synthase (mtNOS) produces nitric oxide (NO) from L-arginine, in this work we found that there is not synthesis of NO in the absence of superoxide dismutase (SOD), suggesting that nitroxyl (NO^-) or a related molecule is produced by mtNOS and then converted to NO by SOD. Besides oxidizing L-arginine, mtNOS reduces various electron acceptors, including cytochrome c and tetrazolium salts (NBT). We also found that NADPH-dependent diaphorase activity of mtNOS does not depend on the presence of Ca^{2+}/CaM. Moreover, this activity catalyzes an SOD-insensitive reduction of NBT and is quenched by diphenyleneiodonium (DPI). Of interest was the finding that cytochrome c reduction required Ca^{2+}/CaM and was blocked by SOD and the specific NOS inhibitor 7-nitroindazole (7-NI). Thus, mtNOS can also catalyze superoxide (O_2^-) formation, primarily at the heme center of its oxygenase domain.

Acknowledgements

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Amino acid derivatives as anti-allergic: synthesis and structure-activity relationship (SAR) of some piprazinyl-amino acid-acrylamide derivatives

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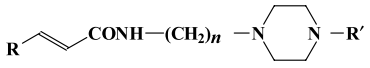
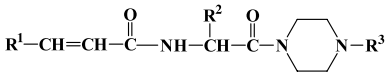
In search for biochemically rationalized anti-allergics, possibly inhibitors of 5-lipo-oxygenase, depending on two action mechanisms of the ready drugs used to treat allergy *e.g.* ''Oxatomide'', which contains piprazine moiety, its anti-allergic effects possess antagonist activities against chemical mediators such as histamine, serotonine and leukotriens which cause allergy. Also ''Transilate and Amoxamox'', which contain acrylic acid segment, its activity depends on inhibition of mediator release.

In harmony with these findings, Nishikawa et al., synthesized new compounds



[A]

comprising acrylic acid and piprazine segments coupled together with aliphatic chains (Fig. A). In our work, we used amino acids derivatives instead of the aliphatic chain (Fig. B).



[B]

Our approach to use amino acids was based on the fact that several amino acids and peptide derivatives are valuable drugs. Seven compounds have the general formula [B] were synthesized. Thus, cinnamic acid and 2-thiopheneacrylic acid were coupled with a series of the esters hydrochlorides of amino acids namely β-alanine, valine, tyrosine and D-phenylalanine *via* the active intermediates to afford the corresponding acryloylamino acid esters. The later after mild alkaline hydrolysis to afford the corresponding acid derivatives, which were coupled with different piprazine derivatives as methyl-, 4-fluorophenyl-, 4-ethylcarboxylate and 4-hydroxyethylpiprazine to give our targeted products

The anti-allergic activities of the synthesized compounds were measured as:

- Anti-anaphylactic activity (Schultz-Dale reaction).
- Experimental allergic encephalomyelitis (Immunosuppressive properties).
- Inhibition of allogenic transplant rejection.

Results showed very interesting data and many of the synthesized compounds showed high activities. The structure of the obtained products as well as their antiallergic activities will be presented.

The role of "polyamine test" for the evaluation of the clinic and biological correlations in the process of schizophrenia patients treatment

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Correlation between the dynamics of clinical presentations and polyamine levels in blood serum during treatment was examined before treatment beginning, after the active psychotic semiotics recession, and in discharge from the hospital. During treatment the reduction in clinical rates intensity (BPRS score) was observed equally with the decreasing of polyamine levels in patient blood serum. After the active psychotic symptoms recession in patients with shift-like schizophrenia it was appeared positive correlation between the degree of reduction of such symptoms as disorganization of thinking, depressed mood, delusions and jealonsy, and decreasing of putrescine concentration in blood. High extant of the correlation was detected for sprmidine too. The degree of diminishing of many clinical symptoms also correlates positively with declining of polyamine levels. The highest coefficients of multiple correlation were detected between the decreasing of polyamine quantity and such symptoms as disorganization of thinking ($r = 0.827$ $p < 0.001$), anxiety ($r = 0.549$ $p < 0.001$), depressed mood ($r = 0.518$ $p < 0.001$), delusions and jealousy ($r = 0.706$ $p < 0.001$), and decreasing of total amount of steps by BPRS score ($r = 0.683$ $p < 0.001$). During the active psychotic semiotics recession in patients with paranoid schizophrenia the reliable range of correlation between declining of polyamine levels and smoothing of such clinical symptoms as: somatic anxiety ($r = 0.665$ $p < 0.001$), autism ($r = 0.536$ $p < 0.001$), dullness of affect ($r = 0.580$ $p < 0.001$), was observed equally with high correlation between declining of polyamine levels and decreasing of symptom intensity (for anxiety $r = 0.559$ $p < 0.001$; for delusions and jealousy $r = 0.600$ $p < 0.001$). At discharge from the hospital patients with shift-like schizophrenia showed the closest positive correlation between declining of polyamine levels and reducing of such clinical symptoms as emotional isolating ($r = 0.552$ $p < 0.001$), disorganization of thinking ($r = 0.540$ $p < 0.001$), exertion ($r = 0.533$ $p < 0.001$), hallucinatory behavior ($r = 0.503$ $p < 0.001$), motional lethargy ($r = 0.606$ $p < 0.001$), unusual thoughts ($r = 0.529$ $p < 0.001$). For the patients with paranoid schizophrenia the most closest degrees of multiple correlation were detected between declining of polyamine levels and decreasing of such clinical symptoms (by the BPRS score) as guilt feeling ($r = 0.761$ $p < 0.001$), delusions and jealousy ($r = 0.683$ $p < 0.001$), disorganization of thinking ($r = 0.564$ $p < 0.001$), autism ($r = 0.585$ $p < 0.001$), unusual thoughts ($r = 0.565$ $p < 0.001$). At the same time for these patients, like for patients with shift-like schizophrenia, the important contribution into multiple correlation was brought by spermine concentration. At discharge from the hospital for patients with paranoid schizophrenia the most closest correlation was observed between spermidine concentrations and such clinical symptoms as somatic anxiety ($r = 0.708$ $p < 0.001$), anxiety ($r = 0.633$ $p < 0.001$), emotional isolating ($r = 0.506$ $p < 0.001$), motional lethargy ($r = 0.739$ $p < 0.001$), autism ($r = 0.638$ $p < 0.001$), dullness of affect ($r = 0.564$ $p < 0.001$). For patients with shift-like schizophrenia the closest correlation was detected between spermidine levels and clinical symptoms, such as emotional isolating ($r = 0.612$ $p < 0.001$), disorganization of thinking ($r = 0.599$ $p < 0.001$), autism ($r = 0.458$ $p < 0.001$), dullness of affect ($r = 0.584$ $p < 0.001$). Thus, the statistical treatment by correlation analysis of clinical and biochemical data exposed positive correlation between serum polyamine levels and symptoms of patients with schizophrenia.

Carcinogenic and carcinostatic properties of psychotropic medications

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The purpose of this research is to study the biological properties of polyamines' (PA) structural analogs from among psychotropic medications. The objective is to research into the effect of psychotropic medications (polyamines' structural analogs) on polyamines' main metabolic indicators (diamine oxidase – DAO and polyamine oxidase – PAO activity) in tissues with an enhanced mitotic activity. Preliminary results and data available in books on the subject suggest that changes in the mitotic activity of tissues correlate in a certain way with the PA level. Thus, during an intensive cell division (regeneration, tumor and embryo growth) an enhanced polyamines level is observed. This is why PA metabolism can be a suitable experimental target in studying the proliferative activity of tissues and the biological properties of substances that influence that activity. Two different mechanisms of increasing the polyamines levels in tissues with "normally" and "pathologically" enhanced cell proliferation have been described earlier. Thus, in the

hepatoma tissues and as a result of hepatocancinogenesis caused by diethylnitrosamine, the increase is due to a sharp reduction or a total loss of DAO and PAO activity in a greater degree than as a result of an enhanced ornithine decarboxylase activity. A higher PA level in a regenerating liver depends on a sharp increase in the synthesis rate of these substances since the rate of their disintegration by way of an oxidative desamination remains constant. Hence, substances inhibiting an oxidative desamination of PA are likely to manifest carcinogenic properties. And vice versa, chemical compounds activating the process of oxidative disintegration of putrescine and PA may have an antitumoral potential.

The biological properties of medications (psychotropic agents) (PA structural analogs) have been studied: I – Ridazine(thioridazine): 10-[2-(1-methyl-2-piperidazinyl)ethyl]-2-(methylthio)-10H-phenothiazine hydrochlorid, II – Mageptyl (thioproperazine): N, N-dimethyl-10-[-(4-methyl-1-piperazinyl)propyl]-10H-phenothiazine-2-sulfonamide, III – Tizercine (levomepromazine): 2-methoxi-N,N-beta-trimethyl-10H-propanamine, IV – Chlorpotixen hydrochlorid: (Z)-3-(2-chlor-9H-thioxanten-9-ilyden)-N,N-dimethyl-1-propanamin hydrochlorid, V – Triphtazine (trifluoropyrazine): 10-[3-(4-methyl-1-piperazinyl)propyl[-2-(trifluoromethyl)-10H-phenotiazine, VI – Aminozine: 2-chlor-N,N-dimethyl-10H-phenothiazine-10-propanamine, VII – Fluphenazine: 4-[3-[2-(trifluoromethyl)-10H-phenotiazine-10-yl]propyl]-1-piperazinylethanol, VIII – Azaleptine (klozapine), IX – Melipramine, X – Amitriptyline, XI – Aeglonile, XII – Haloperidol.

The effect of substances on PA disintegration has been quantitatively assessed on a model cell-free testing system of tissues with a retained system of controlling the rate of the cell proliferation: the rat regenerating liver postmitochondrial fraction. The carcinogenic and carcinostatic properties of chemically modified polyamines analogs have been assessed by character and level of their effect on the rate of PA disintegration during incubation. The total quantity of the substance for the whole research cycle is 2 μM. This small quantity is a favorable distinction of this method as compared to other approaches to this problem. The total testing time does not exceed several hours. These advantages allow for use of this testing system in primary collection of low-toxic substances with a potential antiproliferative activity and in forecasting carcinogenic properties of chemical compounds.

Oxidative desamination of putrescine has been activated by all substances, that of spermidine – by all substances except VIII and that of spermine – only by III, IV, V, VIII and XI. The medications have been ranged according to the results in the ascending order of their carcinostatic properties: IX < VIII < VII < VI < II < XII < XI < X < V < III < IV < I. Any excess over the control figures has been expressed as a percentage. In total it has enabled assessment of the effect of each medication on the diaminoxidase and polyaminoxidase activity in general. Compounds I, II, IV and V that have substantially activated the process of oxidative desamination of putrescine and PAs can already be considered at this stage of testing as potential carcinostatics. Standardization of the used method of testing chemical compounds would allow for monitoring carcinogenic and carcinostatic properties of the existing drugs and forecasting the quality of new medications.

Role of tissue protein synthesis in urea synthesis of rats given proteins of different quality

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The purpose of present study was to determine whether the regulation of urea synthesis is mediated through changes in supply of amino acids by protein synthesis and whether the concentration of activities of amino acid catabolizing enzymes regulate urea synthesis when the dietary protein quality is manipulated. Experiments were done on three groups of rats given diets containing 10 g gluten, 10 g casein or 10 g whole egg protein/100 g diet for 10 d. The urinary excretion of urea, and the liver concentrations of glutamate, serine and alanine increased with a decrease in quality of dietary protein. The fractional and absolute rates of protein synthesis in tissues declined with the decrease in quality of dietary protein quality. The activities of hepatic amino acid catabolizing enzymes was not related to urea excretion under these conditions. These results suggest that the lower protein synthesis seen in tissues of rats given the lower quality of protein is likely to be one of the factors to increasing the supply of amino acids and stimulating urea synthesis.

In vivo production of taurine in rats and mice injected with L-cysteinesulfinate

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In mammalian tissues, L-cysteine is metabolized to sulfate and taurine, two major final metabolites of cysteine sulfur. The oxidation state of sulfur atom of these metabolites is +6 and that of cysteine is −2. During the metabolism of cysteine to sulfate and taurine, sulfur atoms are successively oxidized from −2 to +6. It is well known that sulfur is an active element and reacts readily with oxygen, namely, it is easily oxidized. Thus, the metabolites containing sulfur of various oxidation states are supposed to act as antioxidants in mammalian tissues. Therefore, it is important to study the activities of cysteine metabolism.

L-Cysteinesulfinate (CSA) is the key intermediate of the oxidation pathway of cysteine metabolism. CSA is metabolized through 2 pathways, of which the initial reactions are catalyzed by L-cysteinesulfinate decarboxylase and aspartate aminotransferase, producing final metabolites inorganic taurine and sulfate, respectively.

We studied *in vivo* production of taurine, hypotaurine and sulfate (free plus ester) following administration of L-cysteinesulfinate to rats and mice by determining tissue and urinary contents of these metabolites. When 5.0 mmol/kg body weight of CSA was injected to rats (n = 7), increased urinary excretion of taurine, hypotaurine and sulfate in 24 hour urine were 617, 52 and 1,767 μmol/kg, respectively, indicating taurine production was approximately 1/3 of sulfate production under the present conditions.

Increased contents (μmol/g wet tissue) of taurine and hypotaurine in mouse tissues (n = 6) at 60 min after the injection of 5.0 mmol/kg body weight of CSA were: liver, 3.5 and 9.9; kidney, 0.3 and 5.2; heart, 3.7 and 0.2; blood plasma, 0.4 and 0.2; skeletal muscle, negligibly low, respectively. These results indicate that liver is the most active tissue for taurine production, followed by kidney. When hypotaurine was injected to mice, its contents in liver and kidney increased greatly: 18.8 and 12.1 μmol/g, respectively. Taurine injection resulted in three times and twice the original amounts in liver and kidney, respectively. These results indicate that hypotaurine and taurine are easily transported into these tissues.

The effect of cysteine precursors on sulfane sulfur levels and U373 cells proliferation

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A dependency is suspected to exist between the level of sulfane sulfur and the proliferation of cancer cells. The sulfane sulfur level, in turn, is dependent on the level of L-cysteine and on the activity of enzymes

responsible for its conversion to sulfane sulfur-containing compounds, such as 3-mercaptopyruvate sulfurtransferase (MPST), and their utilization (rhodanese). The verification whether N-acetylcysteine and ribose-cysteine (2(R,S)-D-ribo-(1′,2′,3′,4′-tetrahydroxybutyl)-thiazolidyne-4(R)-carboxylic acid), precursors of cysteine, may affect the proliferation of the human astrocytoma cell line U373 through the elevation of the level of sulfane sulfur in these cells is a novel approach to the problem of inhibiting neoplastic cell proliferation. Both N-acetylcysteine (0.25–1 mM concentration in the culture medium) and ribose-cysteine (2–5 mM) increase the level of cysteine and glutathione in U373 cells (6 h–48 h of incubation). The effect of NAC depends on its concentration in the culture medium and on the incubation time; a diminished cell proliferation was observed (0.5 mM, 48 hrs) when the increased MPST activity was accompanied by the increased level of sulfane sulfur. A decreased sulfane sulfur level (1 mM, 12 hrs) accompanied increased cell proliferation. The conditions (concentration and incubation time) allowing for inhibition of U373 cells proliferation for ribose-cysteine were not found. Ribose-cysteine stimulated cells proliferation, what was accompanied by a decreased level of sulfane sulfur, as well as significantly increased glutathione levels and the activity of rhodanese.

Neurobiology

Glutamatergic mechanisms regulating GABAergic inhibitory transmission in the basolateral amygdala

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Temporal lobe epilepsy is the most common form of epilepsy, and one that is often resistant to drug therapy. The amygdala, a temporal lobe structure, plays a central role in the pathogenesis and symptomatology of temporal lobe epilepsy. Yet, current knowledge on the mechanisms regulating neuronal excitability and epileptogenesis in the amygdala is very limited. Such knowledge is necessary for the development of therapeutic approaches that can prevent or treat temporal lobe epilepsy. Neuronal excitability in the brain is determined by the level of excitatory synaptic transmission – primarily glutamatergic – relative to the level of inhibitory synaptic transmission – primarily GABAergic. The balance between excitation and inhibition in the brain is maintained by a number of regulatory mechanisms. Here we demonstrate that in the rat basolateral nucleus of the amygdala (BLA) – which is the amygdala nucleus primarily responsible for the generation of widespread status epilepticus – a subtype of glutamate receptors, namely kainate receptors containing the GluR5 subunit (GluR5-KRs) play a major role in the regulation of neuronal excitability. In addition to participating in synaptic transmission in pyramidal to pyramidal cell and pyramidal cell to interneuron synapses, GluR5-KRs are present on presynaptic terminals of GABAergic interneurons, where they exert a powerful and tight regulation of GABA release. The clinical significance of the function of GluR5-KRs in the BLA is suggested by the findings that a) activation of these receptors induces epileptiform activity in *in vitro* amygdala slices, and epilepsy *in vivo*, and b) topiramate, a structurally novel antiepileptic drug, exerts its anticonvulsant actions, at least in part, by blocking GluR5-KRs. Supported by USUHS-CS70SG.

Alterations of taurine in the brain of chronic kainic acid epilepsy model

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The aim of the study was to investigate changes of taurine in the kainic acid (KA, 10 mg/kg, s.c.)-induced spontaneous recurrent seizure model of epilepsy, six months after the initial KA-induced wet dog shakes (WDS) and seizures. The brain regions investigated were caudate nucleus, substantia nigra, septum, hippocampus, amygdala/piriform cortex and frontal, parietal, temporal and occipital cortices. In control animals the highest taurine levels were seen in caudate nucleus (964.0 ± 46.7 ng/mg wet tissue weight) and amygdala/piriform cortex (922.0 ± 15.3 ng/mg wet tissue weight) followed by cortical regions and hippocampus and the lowest levels were seen in the septum (570.5 ± 29.5 ng/mg wet tissue weight) and the substantia nigra (485.5 ± 13.8 ng/mg wet tissue). In KA rats with spontaneous recurrent seizures and WDS six months after KA injection increased taurine levels were found in the caudate nucleus (162.5% of control; $p < 0.001$) and the hippocampus (126.6% of control; $p < 0.01$), while reduced taurine levels were seen in the septum (78.2% of control; $p < 0.05$) and normal taurine levels were measured in the other brain regions. In KA rats with only rare focal seizures and WDS six months after KA injection increased taurine levels were found in the hippocampus (125.4% of control; $p < 0.05$). In summary, spontaneous recurrent seizures in KA-rats were associated with significantly increased taurine levels in the caudate nucleus and this alteration could be involved in the modulation of seizure activity.

Striatal metabotropic glutamate receptors as a target for pharmacotherapy in Parkinson's disease

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Parkinson's Disease (PD) is a common neurodegenerative disorder characterized by the loss of dopamine (DA)-containing neurons in the substantia nigra pars compacta. The symptoms are resting tremor, slowness of movement, rigidity and postural instability. Evidence that an imbalance between dopaminergic and cholinergic transmission takes place within the striatum led to the utilization of DA precursors, DA receptor agonists and anticholinergic drugs in the symptomatic therapy of PD. However, upon disease progression the therapy becomes less effective and debilitating effects such as dyskinesias and motor fluctuations appear. Hence, the need for the development of alternative therapeutic strategies emerged.

Several observations in different experimental models of PD suggest that blockade of excitatory amino acid transmission exerts antiparkinsonian effects. In particular, recent studies have focused on metabotropic glutamate (mGlu) receptors. Drugs acting on group I and II mGlu receptors have indeed proven useful in ameliorating the parkinsonian symptoms in animal models of PD and therefore might represent promising therapeutic targets. This beneficial effect could be due to the reduction of both glutamatergic and cholinergic transmission. A novel target for drugs acting on mGlu receptors in PD therapy might be represented by striatal cholinergic interneurons. Indeed, the activation of mGlu2 receptors, highly expressed on this cell type, is able to reduce calcium-dependent plateau potentials by interfering with somato-dendritic N-type calcium channel activity, in turn, reducing ACh release in the striatum. Similarly, the blockade of both group

I mGlu receptor subtypes reduces cholinergic interneuron excitability, and decreases striatal ACh release. Thus, targeting mGlu receptors located onto cholinergic interneurons might result in a beneficial pharmacological effect in the parkinsonian state.

Regulation of glutamate level in rat brain through activation of glutamate dehydrogenase by *Corydalis ternata*

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When treated with protopine and alkalized extracts of the tuber of *Corydalis ternata* for one year, significant decrease in glutamate level and increase in glutamate dehydrogenase (GDH) activity was observed in rat brains. The expression of GDH between the two groups remained unchanged as determined by western and northern blot analysis, suggesting a post-translational regulation of GDH activity in alkalized extracts treated rat brains. The stimulatory effects of alkalized extracts and protopine on the GDH activity was further examined *in vitro* with two types of human GDH isozymes, hGDH1 (house-keeping GDH) and hGDH2 (nerve-specific GDH). Alkalized extracts and protopine activated the human GDH isozymes up to 4.8-fold. Most distinctive differences in their sensitivity to stimulation by protopine and alkalized extracts between hGDH1 and hGDH2 was observed in the presence of ADP. Studies with cassette mutagenesis at ADP-binding site showed that hGDH2 was more sensitively regulated by ADP than hGDH1 on the activation by *Corydalis ternata*. Our results suggest that prolonged exposure to *Corydalis ternata* may be one of the ways to regulate glutamate concentration in brain through the activation of GDH.

Regulation of the hypothalamic neurohypophysial system by taurine and GABA under stress: selective control of vasopressinergic versus oxytocinergic neurons

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Magnocellular vasopressin (AVP) and oxytocin (OXT) containing neurons of the hypothalamic supraoptic (SON) and paraventricular nuclei comprise the hypothalamic-neurohypophysial system (HNS), which is crucially involved in the regulation of body fluid and electrolyte homeostasis. However, recent studies suggest also an activation of HNS neurons during stress. This holds particularly true for their ability to release AVP and OXT not only from axon terminals into the general circulation but also from somata and dendrites into the extracellular fluid of the SON. We investigated in a series of experiments the role excitatory and inhibitory amino acids play in controlling the secretory activity of HNS neurons. To do that microdialysis was used to monitor amino acid release within the SON in adult male Wistar rats. The animals were exposed to different stressors and treated with antagonists blocking the interaction of either taurine or GABA with their postsynaptic receptors. Our data demonstrate that glutamate stimulates the release of AVP and OXT from HNS neurons both into blood and within the brain. Taurine was found to selectively inhibit the release of AVP into both compartments whereas GABA that of OXT. We further administered a specific taurine antagonist directly into the SON and monitored the plasma ACTH concentration – a measure for the activity of the endocrine stress axis – before, during and after a defined stressor exposure. This treatment resulted in a significantly reduced ACTH secretion. Taken together, our data imply that in the SON taurine and GABA selectively control the secretory activity of AVP and OXT neurons, respectively, under stress. Furthermore, taurine seems to control at the level of the SON the endocrine stress axis activity.

Are astrocytes vulnerable to intracellular depletion of transported glutamate?

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In brain, glutamate transport (GT) maintains low, non-toxic, extracellular concentrations of the neurotransmitter glutamate and feeds intracellular metabolic pathways. We report here that altered GT can induce astrocyte death not because of the raise in extracellular glutamate but by depleting an intracellular pool of glutamate, essential for glutathione synthesis. L-trans-pyrrolidine-2,4-dicarboxylate (PDC), a substrate inhibitor of GT, induced a delayed loss of differentiated striatal astrocyte in culture, preceded by an extracellular glutamate increase, a depletion of total intracellular glutamate, a decrease in glutathione contents and a dramatic raise in reactive oxygen species. The death is mediated by oxidative stress, as protection was provided by antioxidants, but not through the increase in extracellular glutamate since L-glutamate addition decreased cell death and prevented glutathione depletion. Failure of the glutamate/cystine exchanger or cystine deprivation was less potent than PDC to elicit astrocyte death, so that altered GT-mediated gliotoxicity cannot be attributed to impaired cystine transport solely. Among glutamate analogs, only L-aspartate, which is both transportable and metabolizable into glutamate, offered protection. The protective effects of glutamate were also mimicked by inhibiting glutamine synthetase that uses transported glutamate for glutamine synthesis. Moreover, astrocyte death was closely correlated with the potency of different GT inhibitors to induce glutamate release by heteroexchange. Therefore, the death-inducing event is likely to be the depletion of a compartmented intracellular pool of glutamate specifically fuelled by GT and crucial for glutathione synthesis. This is the first mechanistic explanation for astrocyte death in conditions of reverse GT such as occurring in ischemia.

Acute phase proteins as potential plasma biomarker in schizophrenia identification

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In this study, we attempted to identify plasma biomarkers of schizophrenia by analyzing the plasma of schizophrenic patients with technology of proteomics in order to investigate the etiology of the disease. We found that the expression of haptoglobin (Hp) alpha chain, α1-antitripsin, serum amyloid P-component, α1-microglobulin, antithrombin III and vitamin D binding protein were significantly higher in the plasma of schizophrenic patients in comparison with healthy controls. Four differential expression proteins belonging to the family of positive acute phase proteins (APPs) were all up-regulated in patients. These 4 APP genes are localized in or nearby positive linkage chromosomal regions previously identified from genome-wide linkage studies. The evidence from proteomic and genomic levels respectively indicate that acute phase reaction may be an aetiological agent of the pathophysiology of schizophrenia, and not just an accompanying symptom. In conclusion, positive APPs might become biomarkers for schizophrenic diagnosis and prognosis.
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Effects of perinatal asphyxia on cns plasticity studied with *in vitro* and *in vivo* models

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While the clinical relevance of perinatal asphyxia is well established, the pre-clinical research is still at an exploratory phase, mainly because of a lack of consensus on a reliable and predictable model. We have developed a model for investigating the issue, considering that model (i) mimics well some relevant aspects of human delivery; (ii) is largely non-invasive; (iii) allows to study with the same preparation short- and long-term consequences of the insult, and (iv) is highly reproducible among laboratories.

Asphyxia is induced by immersing foetuses-containing uterine horns removed from ready-to-deliver rats into a water bath. After different periods, the animals are removed from the water bath, delivered from uterine and amniotic tissue and stimulated to breathe on a warming pad pending a first behavioural assessment and/or sacrificed for biochemical analysis. The pups are given to surrogate dams and sacrificed after three or seven days for preparing organotypic cultures, or left to develop for further *in vivo* experiments. Sibling caesarean-delivered pups are used as controls. We have observed that the dopamine systems are particularly vulnerable to perinatal asphyxia, with different effects on different regions, to be seen long after development, whether investigated *in vitro* or *in vivo*.

The effect of perinatal asphyxia on brain development is studied with organotypic cultures from substantia nigra (SN), neostriatum (Str) and neocortex (Cx), or from hippocampus. Morphological features and cell viability are recorded during *in vitro* development, and at the age of one month, the cultures are fixed and treated for immunocytochemistry.

Organotypic triple cultures (SN, Str and Cx) from control animals survive well, but those from asphyxia-exposed animals show (i) a decreased *in vitro* survival, (ii) a decreased number of NR1-positive neurons in substantia nigra, and (iii) a decreased number of secondary to higher level branching of TH-positive neurites, illustrating the vulnerability of the dopaminergic systems to perinatal asphyxia. In addition, (iv) NOS containing neurons are increased in cultures from asphyxia-exposed animals, suggesting an increase of nitric oxide levels following severe perinatal asphyxia.

Hippocampus cultures from control animals also develop well, preserving the layering and regional subdivisions shown *in vivo*, and only few dying cells are observed when treated for the viability test. Immunocytochemistry has revealed, however, (i) a decreased amount of MAP-2 positive cells in cultures from asphyxia-exposed animals, and (ii) an increase in cellular proliferation assayed with BrdU labelling, suggesting a compensatory mechanism for the long-term impairments induced by perinatal asphyxia.

In a series of *in vivo* experiments, pups exposed to asphyxia and controls have been treated with nicotinamide, or saline for three days, from 24 h after birth, attempting to maximize a NADH/NAD+ replenishing effect. We have found that nicotinamide-treated asphyxia-exposed animals show a recovery in several neurotransmitter parameters, reaching monoamine levels closed to those observed in controls. Thus, we are further investigating the possible protection provided by nicotinamide, scaling up the treatment to a first clinical multicenter aleatory study on newborn babies suffering of perinatal asphyxia, coordinated by the Department of Paediatrics-North, Medical Faculty, University of Chile.

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Maurotoxin, a four disulfide bridges scorpion toxin acting on K^+ channels

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Maurotoxin (MTX) is a unique toxin isolated from the venom of the Tunisian chactidae scorpion *Scorpio maurus palmatus*. Compared with conventional three-disulfide-bridged scorpion toxins and two other recently reported toxins, Pi1 and HsTx1 from the venoms of the scorpions *Pandinus imperator* and *Heterometrus spinnifer*, respectively, MTX adopts an unusual disulfide bridge motif: the α-helix is connected by two disulfide bridges to two different strands of the β-sheet instead of connecting the α-helix to the same strand This uncommon toxin displays an exceptionally wide range of pharmacological activity, as it was found to show activity in the nanomolar range on both voltage-gated K^+ channels (Kv1.1, Kv1.2, Kv1.3, and *Shaker* B) and apamin-sensitive small-conductance Ca^{2+}-activated K^+ channels (SK). The structural and pharmacological features of MTX (less than 40 residues, four disulfide bridges, and binding onto K^+ channels) suggest that MTX belongs to a new class of natural K^+ channel blockers structurally intermediate between the Na^+ (60–70 residues and four disulfide bridges) and K^+ channel scorpion toxin families (less than 40 residues and three disulfide bridges). Recently, much attention has been paid to the pharmacological activity of MTX on the Kv1 channels. Like most scorpion toxins, MTX blocks the Kv1 channels by binding in the external vestibule of the pore to block the ion conduction pathway. Although Kv1.1, Kv1.2, and Kv1.3 have a very similar pore structure, they display different pharmacological activity inhibited by MTX, IC_{50} values are 37, 0.8, and 150 nM, respectively. Particularly, it is worth noticing that MTX was described as a potent blocker of Kv1.2 (IC_{50} of 0.8 nM) compared with other known three-disulfide-bridged blockers such as noxiustoxin (IC_{50} of 2 nM) and charybdotoxin (IC_{50} of 14 nM). Thus, MTX can be used as a structural probe to identify the critical residues of the nonconserved pore-forming sequence in the recognition of the Kv1 channels. Therefore, an understanding of the molecular interactions between MTX and the Kv1 channels, will provide insights into the mechanisms underlying the specificity of channel-toxin interaction.

Influence of L-beta-ODAP on $[Ca^{2+}]$i of rat primary motor neuron in relation to neurolathyrism

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3-*N*-oxyalyl-L-2,3-diaminopropanoic acid (L-beta-ODAP) is an 2-amino-3-hydroxy-5-methylisoxazol-4-propionate (AMPA)-type glutamate receptor (AMPA-R) agonist contained in grass pea (*Lathyrus sativus* L.). Overconsumption of this pea sometimes causes human neurolathyrism. The mechanism of the toxicity of L-beta-ODAP has not been fully clarified. Intracellular Ca^{2+} ($[Ca^{2+}]$i) is one of the most important cellular signals in all neurons. However, it also causes motor neuron death in the case of overload. To clarify the action of L-beta-ODAP on motor neuron $[Ca^{2+}]$i dynamics, Ca^{2+}-imaging using Ca^{2+}-senseitive

dye Fluo-3 was performed after acute L-beta-ODAP treatment, and compared with (*S*)-AMPA using rat primary motor neuron from spinal cord. L-beta-ODAP dose-dependently evoked ∼2 fold increase in $[Ca^{2+}]$i that persisted >20 min. The precise pharmacological analysis of this $[Ca^{2+}]$i rise clarified following results. AMPA-R antagonists completely abolished $[Ca^{2+}]$i triggered by both agonists, whereas either *N*-methyl-D-aspartate or group I metabotropic Glu receptor antagonists lowered $[Ca^{2+}]$i partially. Blockers revealed the involvement of P/Q type Ca^{2+} channel in the $[Ca^{2+}]$i by L-beta-ODAP at the early phase much more than L- or N-type channels. On the other hand, $[Ca^{2+}]$i rise of (*S*)-AMPA was largely dependent on L-type channel. The $[Ca^{2+}]$i rise of L-beta-ODAP required extracellular Ca^{2+}, however small but reproducible $[Ca^{2+}]$i increase was left in the presence of thapsigargin/EGTA suggesting the involvement of other organelles in this response than ER. Taken together, L-beta-ODAP showed a characteristic $[Ca^{2+}]$i profiles that might have relationship to the neurolathyrism.

mGluR5 antagonist MPEP does not induce neuronal death in immature rat brain in contrast to NMDA antagonist MK-801

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MPEP, a selective metabotropic glutamate receptor (mGluR5) antagonist, and MK-801, a high affinity noncompetitive NMDA receptor antagonist exhibit marked anticonvulsant effect in adult as well as immature rats. On the other side, MK-801 induces neuronal death in immature rodent brain, therefore we decided to study if MPEP exhibits similar negative effect.

Wistar male rats 12, 18, and 25 days old were studied. MPEP (40 mg/kg) and MK 801 (1 mg/kg) freshly dissolved in isotonic saline were injected intraperitoneally. Control siblings received an equal volume of physiological saline.

For morphological study 4–8 rats formed individual age and drug groups. Fluoro-Jade B (FJB), was used to visualize neuronal loss. Rats were perfused 24 hours after drug or saline administration. Adjacent coronal sections (50 μm) were used for FJB and Nissl staining. Histological evaluation was focused on cerebral cortex, thalamus and hippocampus, i.e. regions, where mGluR5 are present.

To control the effect of the two drugs spontaneous EEG was monitored in groups of 8–10 rats at all three developmental stages. Control EEG was registered for 10 min and then MPEP (40 mg/kg) or MK-801 (1 mg/kg) were administered and recording continued for 24 hr.

MPEP induced a significant increase of slow wave activity (frequency < 2 Hz) for at least one hour. MK-801 administration did not induce a marked change of EEG activity.

MPEP did not induce neurodegeneration in the three studied structures. In contrast, numerous degenerating neurons were observed after MK-801 administration in 12-day-old rats; this effect decreased with age.

Chronic intake of caffeine during gestation modulates metabotropic glutamate receptors in mothers and fetusses rat heart

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Glutamate is the main excitatory neurotransmitter in CNS that is implicated in learning and memory processes. Metabotropic glutamate receptors (mGluRs) are G-protein coupled receptor classified into mGluI, II and III types. mGluRI receptors are coupled, though Gq/11 protein, to phospholipase C (PLC) activity. A high concentration, glutamate is excitotoxic. To avoid excitotoxic damage, cells increase adenosine release, which acting through A_1 receptors inhibits glutamate release. Stimulatory effects of Caffeine are mediated by adenosine receptor blockade. We have previously described that caffeine chronically ingested during gestation causes down-regulation of A_1 receptors in both mothers and fetuses rat brain. Caffeine also modifies mGluRs in rat brain. The aim of the present work was to analyze whether chronic intake of caffeine during gestation modulates mGluRs in both mothers and fetuses heart. Results show that caffeine intake causes a decrease on total mGlu receptor numbers in mothers heart without significant alterations on receptor affinity. A decrease on $G_{q/11}$ protein was also observed. PLC β_1, the main isoform coupled to mGlu receptors, was not significantly altered. Moreover, non significant differences on basal or PLC-stimulated activity were observed in mothers. In fetuses heart, a significant decrease on total mGlu receptors number without variation on affinity was also observed. Nevertheless, in this immature tissue it was detected a loss of PLC stimulatory effect exhibited by mGluRs agonist. Therefore, chronic caffeine intake during gestation causes a down-regulation of mGlu receptors in both mothers and fetuses rat heart suggesting *in vivo* cross-talk mechanism between both adenosine and glutamate receptors.

Preconditioning and neurotrophins: neuronal resistance to epileptic activity

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Prolonged hypoxia secondary to status epilepticus may lead to serious neuronal dysfunction, sublethal injury or cell death in the cortex. The hippocampus is a brain region critically involved in memory formation. Functional damage is derived from delayed neuronal cell death, a hallmark of hypoxic damage. Overactivation of the N-methyl-D-aspartate (NMDA) receptor subtype and neuronal apoptosis are thought to be crucial components in this neuropathological disorder. Key initiators and executioners of apoptosis and modulators of apoptosis such as neurotrophins may have important roles in delayed neuronal cell death induced by hypoxia. Brain-derived neurotrophic factor (BDNF), a neurotrophin that is highly expressed in the hippocampus, is critical to neuronal survival and maintenance by suppressing pro-apoptotic while increasing anti-apoptotic proteins. Thus, the balance between pro-apoptotic and anti-apoptotic proteins determines neuronal fate. We showed that subtoxic concentrations of NMDA protect vulnerable neurons against glutamate-mediated excitotoxicity in cultured hippocampal neurons through a BDNF autocrine loop that involves the immediate release of BDNF and a later increase in exon 4-specific BDNF mRNA levels. Surprisingly, deletion of the 22 bp intervening sequence (IS) bridging the CRE and NF-κB binding sites significantly enhanced basal expression, indicating that a repressor may bind to and act at the IS. A decoy prepared from the IS increases neuronal survival and exon-4 BDNF mRNA levels. We show that Sharp-2, a basic-helix-loop-helix (bHLH) transcription factor, specifically binds to the IS. This novel discovery suggests that Sharp-2 may play an important role in neuronal survival by

regulating the endogenous expression of BDNF and may lead to the development of targeted treatments to the hippocampus.

ERK phosphorylation and FosB expression are associated with the development of dyskinesia following L-DOPA treatment of hemiparkinsonian mice

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Dyskinesias represent a major complication in the treatment of Parkinson's Disease. We have developed a model of L-DOPA-induced dyskinesia in mice that reproduces the main clinical features of dyskinesia in humans. Dyskinetic symptoms were triggered by repetitive administration of L-DOPA (25 mg/kg, twice a day, for 21 days) in unilaterally 6-OHDA lesioned mice. Dyskinetic symptoms appear towards the end of the first week of treatment and are associated with L-DOPA-induced changes in ΔFosB and prodynorphin expression in direct striatal output neurons. In addition, L-DOPA induces activation of ERK1/2 in the dopamine-depleted striatum. All of these changes occurred after acute L-DOPA but were dramatically increased with chronic L-DOPA. Interestingly, elevated FosB/ΔFosB expression is distributed exclusively within completely lesioned regions of the striatum, displaying an inverse correlation with remaining dopaminergic terminals. Following acute L-DOPA treatment, FosB expression occurs in direct striatal output neurons, while chronic L-DOPA induces FosB expression in NOS-positive striatal interneurons as well as direct striatal output neurons. This model provides a system in which genetic manipulation of individual genes can be used to elucidate the molecular mechanisms responsible for the development and expression of dyskinesias. Funded by Ministerio de Educación y Ciencia, SAF04/864, GEN2003-C06-02/NAC; Fundación la Caixa; and Ministerio del Interior y FIS RTA, G03/05, Spain.

Stress-protective activity of synthetic corticotropin-like peptide leucorticotropin

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The corticotropin-like octapeptide GKVLKKRR (termed as leucorticotropin), which corresponds to the amino acid sequence 81–88 in the precursor of human interleukin-1alpha, was synthesized. The synthesis was carried out on a Vega Coupler C250 automatic peptide synthesizer (USA) and purified by preparative reverse-phase chromatography (Gilson chromatograph, France) on a Waters SymmetryPrep C18 column (19 × 300 mm; Malva, Greece).

We demonstrated that intramuscular and intranasal injections of leucorticotropin were able to normalize the synthesis and secretion of corticosterone in the adrenal cortex, as well as the level of histamine and the activity of diaminoxidase in the myocardium of rats exposed to heat or cold shock. However, when injected to rats under normal conditions, this peptide did not affect any of the above activities.

[^{3}H]leucorticotropin with a specific activity of 22 Ci/mmol has been prepared by the high-temperature solid-state catalytic isotope exchange (HSCIE) reaction. Receptor binding studies of [^{3}H]leucorticotropin showed that it binds with high affinity and specificity to the corticotropin receptor on rat adrenal cortex membranes. Additionally, our data suggest that leucorticotropin is an antagonist of the corticotropin receptor.

Behavioural, pharmacological and biochemical properties of an anticonvulsant agent

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Epilepsy is one of the most common neurological diseases, affecting up to 2.5% of the human population, and its treatment is often unsatisfactory, implying the continuous need for new drugs. Carbamazepine (CBZ) is a reference antiepileptic drug (AED), while Nefopam (NEF) is a known analgesic that has recently been shown to be effective in controlling both neuropathic pain and convulsions in rodents. We compared these two compounds for their capability to protect: 1) from excitotoxicity in cerebellar neuronal cultures and 2) from maximal electroshock (MES)-induced convulsions and death in mice. Both CBZ and NEF were effective in preventing both excitotoxicity signs and neurodegeneration following exposure to 5 μM veratridine for 30 min and 24 h respectively. Concentrations providing full neuroprotection were 500 μM CBZ and 50 μM NEF, while the concentration providing 50% neuroprotection was 200 μM for CBZ and 20 μM for NEF. Neither NEF nor CBZ reduced excitotoxicity following direct exposure to glutamate. Culture exposure to either CBZ or NEF resulted in progressive concentration-dependent neurodegeneration which also depended upon the length of the exposure. The non-toxic concentration was estimated at 100 μM for CBZ and 10 μM for NEF. The toxic effect of NEF (25 μM) was prevented by the presence of the voltage-sensitive L-type calcium channel agonist BayK8644 (2 μM). On the other hand, BayK8644-induced cGMP increase was significantly reduced by concentrations of NEF higher than 25 μM.

In mice, both NEF(20 mg/kg i.p) and CBZ (10 mg/kg i.p) fully prevented MES-induced death, although NEF was significantly more effective than CBZ in preventing MES-induced convulsions. Further biochemical and behavioural data will be discussed.

The somatotrophic axis in the brain and its impact on cognitive functions – Growth hormone and insulin-like growth factor-1 interact with excitatory amino acids in hippocampus

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In recent years the involvement of the growth hormone (GH)/insulin-like growth factor-1 (IGF-1)in brain function has received attention. The psychological improvements seen in GH deficient (GHD) patients following GH replacement therapy and the observation suggesting that GH

may cross the blood-CSF-barrier combined with the identification of specific receptors for the hormone in brain tissues (Nyberg, 2000) have open new aspects for the hormone as a therapeutic agent. Of particular interest is that clinical studies have suggested the hormone to promote learning and memory processes in GHD patients (Burman and Deijen, 1998). Also from animal studies GH is known to influence the N-methyl-D-aspartate (NMDA) receptor-system in the hippocampus, an essential part for long-term potentiation (LTP) formation, which is highly involved in memory acquisition (Le Grevés et al., 2002). A similar effect was recently demonstrated for IGF-1 (Le Grevés et al., 2005). In order to further explore the beneficial effect that GH may have on cognitive functions we examined the effect of the hormone in two groups of male rats suffering from multiple hormone deficiencies as a result of hypophysectomy (Hx). One group of animals was treated for 10 days with daily injections of GH, whereas the other group serving as control received saline under identical condition. The cognitive functions were appraised through performance in the Morris water maze. The GH-treated group exhibited a significantly better performance in the spatial memory task on the second and third trail day compared to the saline-treated group of animals. Prolonged training abolished the difference between the groups and the controls performed equal to the GH-treated rats. After completion of behavioral studies, the expression of the gene transcripts of the NMDA receptor subunits, NR1, NR2A and NR2B as well as the postsynaptic density-95 protein (PSD-95) was analyzed by Northern blot analysis in the hippocampus. Our studies reveal the existence of a positive relationship between the expression levels of the NMDA receptor subunit messages and learning abilities in the Hx rats and that this relationship was improved following GH administration.

Constitutive lack of neuronal nitric oxide synthase affects the hypothalamic vasopressinergic system under resting conditions and upon swim stress in mice

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Arginine-vasopressin (AVP) is secreted into the blood from axon terminals of magnocellular neurones originating in the hypothalamic supraoptic (SON) and paraventricular nuclei. Here we investigated the effect of nitric oxide (NO) on this magnocellular AVP system under resting conditions and in response to forced swimming in wild type (WT) and neuronal NO synthase (nNOS) KO mice. AVP mRNA basal levels in the SON of WT mice were robustly enhanced after swim stress, whereas AVP plasma values remained unchanged at any time point investigated (i.e. 5 min, 15 min and 60 min after stressor onset). Whereas KO animals displayed AVP mRNA levels higher than those of WT mice under basal conditions, mRNA levels were significantly lower in response to forced swimming compared with the WT stressed group. Furthermore, in KO mice AVP values were significantly lower 15 min after stressor onset than at all other time points investigated.

Taken together, the results of the present study suggest that the chronic absence of nNOS causes a significantly altered transcription activity of AVP neurones both under resting conditions and in response to forced swimming. As the increased AVP mRNA levels are not paralleled by similar changes in plasma values, we hypothesise a release of AVP from somata and dendrites of magnocellular neurones into the extracellular space of the SON. Further studies are on the way to elucidate in more detail the interaction(s) between NO/nNOS and AVP to control the neuroendocrine stress response in mice.

An influence of ligands of metabotropic glutamate receptor subtypes on parkinsonian-like symptoms and striatal efferents in rats

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Several data indicate that inhibition of glutamatergic transmission may be important to alleviation of parkinsonian symptoms. However, the use of antagonists of ionotropic glutamate receptors in humans is limited because of their serious side-effects. In contrast, ligands of metabotropic glutamate receptors (mGluRs) seem to be safer and, therefore, more promising. Therefore, the aim of the present study was to search for brain targets of putative antiparkinsonian-like effects of mGluR ligands. In order to inhibit glutamatergic transmission, we blocked group I mGluRs (mGluR1 and mGluR5), or activated groups II (mGluR2/3) and III (mGluR4/7/8).

We found that either systemic or intrastriatal administration of group I mGluR antagonists (mGluR5 – MPEP, MTEP; mGluR1-AIDA) inhibited parkinsonian-like symptoms (catalepsy, muscle rigidity) in rats. MPEP administered systemically and antagonists of mGluR1 (AIDA, CPCCOEt, LY37385) injected intrastriatally reversed also the haloperidol-increased proenkephalin (PENK) mRNA expression in the striopallidal pathway. Similarly, ACPT-1 – an agonist of group III mGluRs (with a preference for mGluR4) administered into the striatum, globus pallidus or substantia nigra inhibited the catalepsy induced by haloperidol. In contrast, agonists of group II (2R,4R-APDC, DCG-IV) administered intrastriatally or into the lateral ventricle reduced neither PENK expression nor the above-mentioned parkinsonian-like symptoms. Moreover, a mixed mGluR8 agonist/AMPA antagonist – (R,S)-3,4-DCPG evoked catalepsy and enhanced both the catalepsy and PENK expression induced by haloperidol.

The present results seem to indicate that antagonists of group I mGluRs or preferential agonists of mGluR4 may possess antiparkinsonian properties, and point at the striopallidal pathway as a potential target of therapeutic intervention.

GABA-CCK-glutamate interactions in the rat amygdala and its relevance to anxiety

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It is accepted that amygdala plays a role in fear and anxiety. Work from several laboratories have indicated that in the brain glutamate and CCK have an overall ansiogenic role whereas GABA has anxiolytic effects. Work from our laboratory have shown that in slices from several brain regions CCK-8S increases the K^+-stimulated release of ^{3}[H]-GABA and Lanza and Makovec have shown that in the ventral striatum glutamate neurotransmission seems to mediate this effect. Now, we have studied whether in the rat amygdala CCK-GABA-glutamate interactions do also exist by looking at the K^+-stimulated release of ^{3}[H]-GABA. It was found that CCK-4 enhanced in a dose-dependent manner the K^+ release of ^{3}[H]-GABA. Moreover, it was also found, that these effects were CCK-B receptors-dependent since CR 2945, a CCK-B antagonist, prevented such effects. Finally, it was found that DNQX, a potent, non-NMDA glutamate antagonist blocked also the effects of CCK-4 suggesting that the CCK-4-induced enhancement of the K^+-stimulated release

of 3[H]-GABA was mediated by the activation of glutamate receptors. Surprisingly, CCK-8S another CCK-B receptor ligand failed to affect the K^+-stimulated ^{3}[H]GABA release. Our results indicate that there are important CCK-GABA-glutamate interactions in the rat amygdala which may have an important role in anxiety and might indicate the existence within this region of different subtypes of CCK-B receptors. Work supported by grant IN221805 from Dirección General de Asuntos del Personal Académico, Universidad Nacional Autónoma de México.

The role of metabotropic glutamate receptors in animal models relevant for schizophrenia

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Both NMDA and mGluR5 have been implicated in schizophrenia. High affinity, uncompetitive NMDA receptor antagonists e.g. phencyclidine can trigger psychotic symptoms in humans. Recent preclinical studies demonstrated that the antagonist of mGluR5, MPEP, augmented behavioral effects indicative for psychotomimetic-like activity produced by uncompetitive NMDA receptor antagonists in animals. Although, *in vitro* studies have also discovered interaction between NMDA receptors and mGluR1 the potential contribution of these receptors in schizophrenia is less known. Therefore, in the present study, we tested whether the selective mGluR5 antagonist, MTEP, and the mGluR1 antagonist, EMQMCM, might induce schizophrenia-like symptoms or potentiate such effects of uncompetitive NMDA receptor antagonist (+)MK-801 in animal models relevant for schizophrenia like locomotor activity and prepulse inhibition (PPI).

Consistently with previous studies, (+)MK-801 (0.1–0.2 mg/kg) enhanced locomotor activity of animals and induced sensorimotor gating deficit. In contrast, MTEP (1.25–5 mg/kg) alone had no effect on spontaneous locomotor activity and PPI, but the dose of 5 mg/kg MTEP augmented the effects of (+)MK-801 in both test. EMQMCM up to 4 mg/kg neither affected locomotor activity or PPI in normal animals nor modified behavioral alterations produced by (+)MK-801. Studies examining the effect of subchronic treatment with MTEP on PPI are currently under investigation.

In conclusion, the present study suggests that neither blockade of mGluR1 nor mGluR5 should induce psychotic symptoms. However, mGluR5 antagonists may potentiate psychotic symptoms evoked by uncompetitive NMDA receptor antagonists or exacerbate such symptoms in schizophrenic patients.

Antidepressant-like effects of group III mGlu receptor ligands

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Several data indicated the involvement of glutamate in the pathophysiology of depression and anxiety. It was well established that compounds which decrease glutamatergic transmission *via* blockade of NMDA or group I metabotropic glutamate receptors (mGlu receptors), produce antidepressant-like action in animal tests and models. Since group III mGluR agonists are known to reduce glutamatergic neurotransmission, by the inhibition of glutamate release, we decided to investigate potential antidepressant-like effects of group III mGluR agonists/modulators, after central administration in rats.

It was found that group III mGluR agonists, ACPT-I (1S,3R,4S)-1-aminocyclo-pentane-1,3,4-tricarboxylic acid, produced a dose-dependent antidepressant-like effect in behavioral despair test after intracerebroventricular injections; PHCCC, (−)-N-Phenyl-7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxamide), a positive allosteric modulator of mGlu4 receptors was not effective, however it markedly enhanced the antidepressant-like effects of low doses of ACPT-I. The effects of ACPT-I in combination with PHCCC were reversed by CPPG (RS)-alpha-cyclopropyl-4-phosphonophenyl glycine), group III mGluR antagonist. Moreover, a dose-dependent antidepressant-like action of group III mGluR agonists RS-PPG ((RS)-4-phosphonophenylglycine), but not HomoAMPA, was found in behavioral despair test, after intracerebroventricular injections.

The obtained results indicate that group III mGluR agonists/modula-modulators produce antidepressant-like effects in behavioral tests, after central administration in rats. The reduction of glutamate release by group III mGluR activation may be a possible mechanism underlying antidepressant-like properties of the tested compounds. In conclusion, the results of our studies indicate that group III mGlu receptor agonists may play a role in the therapy of depression.

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Cognitive aspects in human epilepsy and antiepileptic drugs

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Several landmarks in the clinical evolution of the majority of the epileptic patients have been identified: the severity and frequency of seizures, the genetic background, the age at onset of the epileptogenic insult, the extent of brain lesion, the location of the seizure focus, the antiepileptic treatment and psychosocial factors. From the clinical point of view there are several options for classification: a) some epileptic syndromes are considered benign due to the cessation of seizures with no long-term neuropsychological impairment, even without any antiepileptic treatment in some patients; b) some epileptic syndromes have a clear-cut impairment of cognitive-executive functions possibly related to the epileptogenic topography, the severity of the seizures and the antiepileptic treatments. In some of these cases, patients may benefit from a surgical therapy; c) some epileptic syndromes, characterized by the presence of a neuronal lesion, severe seizures during a long period of life, and neurological and neuropsychological impairment, achieve a complete seizure control. Our aim is to review and discuss the different contribution of each of these epilepsy-related factors in order to understand the clinical features and the evolution of the epileptic syndromes mentioned above.

Caffeine sensitization and cross-sensitization with amphetamine: association to post-synaptic changes in rat striatal neurons

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Acute administration of caffeine, the most widely diffused psychostimulant drug, increases motor behavior, whereas continuous administration produces tolerance. In order to study whether, similar to other psychostimulant drugs, subchronic intermittent administration of caffeine induces sensitization of motor behavior and promotes cross-sensitization to amphetamine effects, rats were treated with caffeine (15 mg/kg i.p.) on alternate days for 14 days. Three days after discontinuation of treatment, a challenge of caffeine (15 mg/kg i.p.) or amphetamine (0.5, 1 mg/kg s.c.) was given. Caffeine induced a sensitized

motor behavioral response, associated with a decrease of adenosine A_{2A} receptor and *zif-268* mRNA levels in striatum and nucleus accumbens. Amphetamine administration produced a higher motor response in caffeine – than vehicle-pretreated rats, associated with a more pronounced increase of *zif-268* mRNA levels in the medial striatum but not in the nucleus accumbens. The potentiation of amphetamine effects was not associated with modifications of amphetamine-induced dopamine release in nucleus accumbens in caffeine-pretreated rats compared to vehicle-pretreated rats. The results demonstrate that intermittent pre-exposure to caffeine sensitizes the motor stimulant effects of both caffeine and amphetamine in rats. Sensitization to caffeine and cross-sensitization to amphetamine appear to be associated to post-synaptic neuroadaptive changes and to be related to the medial striatum rather than the nucleus accumbens.

Role of dopamine and serotonin systems in central sensory filtering processes: Effects of neonatal 6-OHDA lesions and 5-HT1A receptor active compounds on auditory gating in rat

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It has been found that schizophrenics commonly demonstrate abnormalities in central filtering capability following repetitive sensory stimuli. Such deficits in sensory gating can be induced in rodents by administration of psycho-stimulatory drugs, such as amphetamine, and determined using EEG recordings of auditory evoked potentials in response to paired clicks ('auditory gating'). With the use of a paired-stimulus paradigm of auditory evoked potentials, a series of studies were performed to characterize the consequences of developmental plasticity of brain dopamine and serotonin systems as well as the involvement of serotonin $(5\text{-HT})_{1A}$ receptors in central filtering. Modulation of auditory gating by dopaminergic agents was assessed in adult rats that had received intracisternal injections of 6-hydroxydopamine on postnatal day 3. This treatment serves as a model of behavioral hyperactivity coupled with altered response to amphetamine. The lesion results in a selective and profound depletion of central dopamine (DA) when measured at adulthood, concomitant with a hyper-innervation of 5-HT nerve fibers. The adult rats were implanted to record auditory evoked potentials in response to paired clicks delivered 0.5 s apart. Both sham and lesioned animals showed normal gating, which was manifested as a reduction in the response to the second of the paired-click stimuli. Both groups also showed a loss of gating after administration of apomorphine (1 mg/kg, s.c.), while d-amphetamine (1.83 mg/kg, i.p.) disrupted gating only in sham-lesioned animals. In a second set of studies, the effects of two 5-HT_{1A} active compounds, the agonist (*R*)-8-hydroxy-2-(di-n-propyl-amino)tetralin (8-OH-DPAT) and the partial antagonist (*S*)-5-fluoro-8-hydroxy-2-(di-n-propyl-amino)tetralin (UH-301) were investigated. When UH-301 (0.1–5 mg/kg, s.c.) was given to amphetamine-treated animals the loss in gating was attenuated, while UH-301 given by itself did not affect gating. A similar effect on amphetamine-induced loss of gating was seen after 8-OH-DPAT administration (0.05–0.1 mg/kg s.c.). However, 8-OH-DPAT alone also enhanced the gating, as defined by the ratio between the amplitude of the second and the first EEG responses to the clicks.

Taken together, these data show that neonatal loss of DA does not affect auditory gating; however, postsynaptic DA receptor stimulation, even in dopamine-depleted rats, disrupts gating. These results support the conclusion that, in intact animals, the dopamine system plays a critical role in the loss of auditory gating induced by d-amphetamine. However, also 5-HT_{1A} receptors are involved in auditory gating, both under normal conditions and after d-amphetamine-induced loss of the gating. The results confirm the role of the DA system in central sensory filtering processes but also give support for a critical role of the serotonin system. The data obtained suggest that 5-HT_{1A} receptor active compounds may present therapeutic potentials in schizophrenia.

Role of kainate receptors in a developmental rat model of temporal lobe epilepsy

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Epilepsy is a chronic neurological disorder that affects between 0.5 and 2.0% of the adult population. Epilepsy linked to temporal lobe dysfunction (TLE) often originates in childhood but does not manifest until later in life. Current animal models of TLE rely on induction of prolonged motor convulsions (status epilepticus) using either focused electrical stimulation or high doses of chemical convulsants in adult rats. Among the latter, agonists at glutamate receptors, particularly the kainate receptor ligands, kainic acid or domoic acid, are commonly used. While such models share some of the features of clinical TLE, both the induction protocol and certain morphological and neurochemical changes in these models differ from the clinical presentation of the disease.

In a series of recent papers we have reported that extremely low (i.e. selective) doses of kainate receptor agonists administered during a critical window of early postnatal development in the rat result in alterations in neonatal learning in the absence of toxicity. As adults, these rats display seizure-like behaviours in response to novel spatial environments and permanent changes in hippocampal morphology. The current presentation will summarize these previous findings and present new data on changes in spatial learning in young adult rats accompanied by altered patterns of hippocampal circuitry (mossy fibre sprouting) and hippocampal cell loss that closely resemble those seen in human TLE.

Plant Amino Acids

Biochemical characterization of maize high-lysine mutants

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Cereal seeds are deficient in lysine. The genetic manipulation of plant has shown that in order to obtain high-lysine cereal crops, biosynthesis and catabolism of lysine must be altered. Our group has recently isolated the key enzymes of lysine metabolism from sorghum high-lysine seeds and the activities of the enzymes involved in lysine catabolism were shown to be dramatically reduced. Analysis of several maize mutants has shown that some of the high-lysine content of some of the mutants cannot be explained by alteration in lysine catabolism. However, it is clear that the high-lysine trait is due to an altered lysine metabolism and distribution of the storage proteins of the seeds.

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Analysis of transcription factors under sulfur-deficiency stress in *Arabidopsis thaliana*

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Transcriptome analysis, using Affymetrix ATH1 arrays and a real-time reverse transcription-PCR platform for more than 1,400 transcription factors, was performed to identify processes affected by sulfur deprivation and replenishment. One-week-old Arabidopsis seedlings, grown in liquid culture in the sulfur-sufficient medium, were transferred to a sulfur-free medium. After 48 h of sulfur deficiency sulfate was re-supplied for 30 min and 3 h. Thiol measurements revealed a strong decrease of glutathione (GSH) and cysteine which indicates effective S-starvation in the sulfur-depleted plants. Transcript analysis thus revealed strong induction of the genes of the sulfur assimilation pathway. Re-addition of sulfate led to rapid and coordinated changes, which reversed the expression pattern of S-affected genes. Among them many genes were identified of which a group of transcription factors responded to the different treatments similarly as S-pathway genes. This indicates a coordinated regulation of S-metabolism. The respective transcription factors are under investigation.

Specific induction of antioxidant enzymes in *Escherichia coli* by herbicides

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There has been for some years a massive use of agrochemicals in order to improve productivity with a severe impact to the environment. Special attention has been given to bioremediation techniques so that plants and microorganisms can be used to detoxify such compounds. The herbicide Acetochlor has been widely used to prevent the germination of both mono and dicotyledon weed species. We have tested the effect of this herbicide on two *Escherichia coli* strains ($\neq$1 and $\neq$2) and one strain of *Morganela providencia*. The *E. coli* and *M. providencia* strains grown under control conditions and in the presence of the herbicide were used for the extraction of soluble proteins. The extracts were analyzed by SDS-PAGE to determine the protein profile and the activity of antioxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD) were determined following non-denaturing PAGE as described by Medici et al. (Functional Plant Biology 31(1): 1–9, 2004). No apparent differences were observed for *E. coli$\neq$1* and *M. providencia* in soluble protein concentration and distribution, but the intensity of some protein bands were altered for *E. coli$\neq$2* grown in the presence of the herbicide. *M. providencia* exhibited CAT and SOD isoenzyme profiles very different from the *E. coli* strains, but the herbicide did not induce any changes in CAT or SOD activities. On the other hand, the *E. coli* strains responded differently to the herbicide. Large increases in total CAT and SOD activities were detected in *E. coli$\neq$1*, with no change in the proportion of the individual isoenzymes. In *E. coli$\neq$2*, there was evidence of an extensive herbicide specific induction of two new CAT and one new SOD isoenzyme, when compared to the untreated control. The results suggest that there are diverse effects of the herbicide Acetochlor, not only between distinct genera of bacteria, but also between different strains of the same species. Furthermore in *E. coli$\neq$2*, there is evidence of an increase in total activity of the two antioxidant enzymes following the herbicide treatment and that the increases are due to the specific induction of new isoenzymes of both CAT and SOD. These results also indicate that the herbicide is inducing the production of reactive oxygen species which are being metabolized in different ways in the strains tested.

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Nutritional improvement of rice (*Oryza sativa* L.) by pathway manipulation

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Major crops, such as cereals and legumes, are low in cysteine and methionine and an attempt to manipulate the biosynthetic pathway is a major interest of molecular plant breeding. The accumulation of these amino acids in crops would increase the nutritional quality. It can be assumed that Met synthesis, accumulation and consumption are under tight regulatory control. In recent years, several key steps have been identified at the molecular level, enabling us to initiate transgenic approaches to engineer the cysteine and methionine content of plants.

Rice is a major crop plant with deficits in S-containing amino acids. In order to understand the physiological, biochemical, and molecular mechanisms of cysteine and methionine biosynthesis in rice, a transformation system for rice (*Oryza sativa* L. cv. Taipei) was established. At first a cultivation and regeneration system was set up. To test the transformation system a construct expressing GUS under the control of an ubiquitin promoter in a pCAMBIA vector derivative was chosen. The transformation process was performed by co-cultivating the embryogenic calli with *Agrobacterium tumefaciens* for 2–3 days and transformed cells were selected on medium containing 50 mg/l of hygromycin. Successful transformation was proved by GUS staining of transformed calli and plants. Now, the established system will be used to express key genes of cysteine and methionine biosynthesis to increase their respective content. Details will be discussed on the poster.

3D structure of the overlapping sulfonylurea and imidazolinone herbicide binding sites in acetohydroxyacid synthase

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The first enzyme in the common pathway for the biosynthesis of valine, leucine and isoleucine in plants and microorganisms is acetohydroxyacid synthase (AHAS). Interest in AHAS has been stimulated by the discovery that it is inhibited by the sulfonylurea and imidazolinone herbicides. The molecular basis of this inhibition has been the subject of much speculation and our determination of the structure of yeast AHAS containing the sulfonylurea chlorimuron ethyl showed that the inhibitor binds at the entrance of a deep funnel that leads to the active site. The herbicide makes multiple interactions with amino acid side-chains in the active site funnel and mutation of most of these residues results in resistance to sulfonylureas. While yeast AHAS is undoubtedly an excellent model of the plant enzyme, there are some puzzling differences as well. For example, the mutation A122V in *Arabidopsis thaliana* AHAS causes only a small (four-fold) decrease in the sensitivity to sulfometuron methyl but the equivalent mutation (A117V) in yeast AHAS decreases sensitivity by nearly 3000-fold. Thus, it is clearly of interest to know the structure of a plant AHAS to advance our understanding of the binding of these herbicides to their natural target. Therefore, we initiated studies on the structure of *A. thaliana* AHAS and have solved the structure in complex with five sulfonylureas (at resolutions of 2.5–2.9 Å), and one

imidazolinone (at 2.8 Å). These results represent the first report of structural data at atomic resolution for a plant protein with a bound commercial herbicide.

Bio-available amino acids in soil under differently managed meadow ecosystems

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Our knowledge about bio-available amino acids in soil is fundamental for evaluation of nitrogen nutrition in different ecosystems as it is long since known that soil amino acids in their ''free state'' can be uptaken by mycorrhizal or nonmycorrhizal plant roots. This interest results from findings in boreal, alpine and arctic ecosystems where conditions are unfavourable to nitrogen mineralization and plant requirements cannot be satisfied by only mineral nitrogen. As soil amino acids pattern is influenced by plant coverage and soil properties we have performed an evaluation of concentrations of 17 bio-available L-amino acids, extracted by 0.5 M ammonium acetate from mineral soil (Ah layer) of long-term moderately mown alpine meadow and eleven years abandoned part of the same meadow. The selection of the stand resulted from the question coming from abandonment of mountain areas of the Czech Republic at the end of the last century, being accompanied by changes in vegetation coverage and organic matter input. Soil type is Gleyic Luvisol at the both plots and the qualitative pattern of bio-available amino acids can be driven especially by vegetation coverage changes. The most abundant amino acids throughout the experiment were glutamic acid and arginin at both meadow plots. No significant ($P > 0.05$) differences between concentrations each of the 17 amino acids on both stands were found out. Higher and statistically ($P = 0.08$) insignificant amount of amino acids N was measured on mown meadow approx. two weeks after mowing treatment.

Use of "omics"-technologies to reveal transcription factors involved in S-metabolism regulation

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Assimilation of sulfate plays a tremendous role in plants because sulfur enters into many cellular processes that are vital for plants such as one-carbon metabolism, protein and hormone syntheses, detoxification processes. Using novel tools of transcriptomics and metabolomics and appropriate treatments based on sulfate depletion, we were able to map RNA and metabolite changes occurring in the earliest phase of sulfur deficiency in the model plant Arabidopsis thaliana. We also demonstrate that these events are independent of the accumulation of a known metabolite (OAS) assumed previously to be the inducer of sulfate starvation response. Looking for key regulatory genes that may control sulfur assimilation by plants, we selected a number of candidate transcription factor genes putatively involved in control of sulfur metabolism. Analysing the impact of the corresponding mutants and overexpressors *in planta* on sulfur and related amino-acid metabolism, we identified 3 genes that clearly have an effect on sulfur assimilation in *Arabidopsis*. Characterization of these mutants is still in progress. One of them is an AP2-Rav family transcription factor that is involved in root sulfate assimilation; another one is a NAC family transcription factor that affects methionine or branch-chain essential amino-acids levels; a third one is a putative protein that may be involved in the mobilisation of glutathione (the major form of sulfur storage involved in detoxification processes) in the roots.

Cadmium induced changes in antioxidant enzymes in coffee cell suspension cultures

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Pollution is a major worldwide problem. Different types of pollutants are affecting different areas of the globe according to the anthropogenic activities, which are indiscriminate and the main reason for the increase in pollution. Cadmium (Cd) is a toxic heavy metal, which normally occurs in low concentrations in soils; however, its concentration can be high in areas that have been subjected to metal contamination. A group of peptides termed phytochelatins (PCs) has been characterised that are capable of binding heavy metal ions, including Cd. Cd is a non-redox metal unable to participate in Fenton-type reactions yielding reactive oxygen species (ROS), but it enhances lipid peroxidation and decrease GSH content, which results indirectly in ROS production. We studied the antioxidant responses of coffee *in vitro* cells to Cd treatment. Cell suspension culture were grown in increasing concentrations of $CdCl_2$ (0–0.5 mM), for up to 12 days. The cells were analysed for tiobarbituric acid reagent substances (TBARS), catalase (CAT), glutathione reductase (GR), superoxide dismutase (SOD), ascorbate peroxidase (APX), guaiacol peroxidase (GPX) and glutathione-S-transferase (GST). The amount of lipid peroxidation was shown to be increased by Cd treatment in the higher concentration, that was monitored by the elevation in the level of TBARS. CAT, GR and SOD activity increased due Cd treatment, mainly in the higher concentrations of the metal. APX activity was higher in the lower Cd treatement, but could not be detected in cells growing in higher Cd concentrations. GST activity increased only in higher Cd treatments and GPX did not show a clear response to Cd treatment. The analysis by native PAGE revealed one CAT isoenzyme and three GR isoenzymes, however one GR isoenzyme is present only in Cd treated coffee cells. The results suggest that only the higher dosage of Cd treatment lead to oxidative stress, but all Cd treatment lead to enzymatic oxidative responses. The main response may be *via* the induction of SOD and CAT activities for the removal of ROS, or by the induction of GR to ensure the availability of reduced glutathione for the synthesis of Cd-binding proteins (PCs).

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Cadmium induced changes in antioxidant enzymes in tobacco cell suspension cultures

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The contamination of environments by heavy metal pollution, mainly by cadmium (Cd), considered one of the more toxic, is generated by mining and industrial activities, and sewage sludge and phosphated fertilizers used in agriculture. The latter contains Cd in its composition

and with continuous agricultural utilization and increase in the heavy metals levels in soils, plants and water sources, have been observed which may contribute to the bioccumulation and biomagnification in the food chain. A group of peptides termed phytochelatins (PCs) has been identified and are capable of binding heavy metal ions, including Cd and detoxifying the cells by the reduction of free ions in the cytosol. However, oxidative damage often occurs due to the generation of oxygen reactive species induced by heavy metals. A class of antioxidant enzymes has been reported for the scavenging of the oxygen reactive species. Plants exposed to heavy metals may exhibit alteration in enzyme activity. The study of such a response may allow the evaluation levels of tolerance, specificity the response of distinct plant species to levels of pollution in the environment. These data may be useful in breeding programs to select tolerant plants. Moreover, tolerant plants may be used in phytoremediation, by reducing the amount of heavy metals in contaminated soils. In this study, it was observed alterations in CAT and peroxidases activities in BY-2 cells and an extensive increase in GR activity. SOD activity was altered in the isoforms analysed. The results suggest that in BY-2 cells the main defence system to Cd stress is variable during the heavy metal exposure time. The synthesis of GSH, which is used in the synthesis of phytochelatins and substrate for GST is evident during the onset of the stress with CAT and peroxidases taking over the antioxidative responses once the stress becomes severe.

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Molecular analysis and control of cysteine and methionine biosynthesis

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Among the proteinogenic amino acids, cysteine (Cys) and methionine (Met) display many essential direct or indirect functions in cellular metabolism. Cys, the end product of the assimilatory sulfate reduction in plants, is formed from O-acetylserine and sulphide while Met, a member of the aspartate family, receives its carbon backbone from aspartate and its sulphur moiety from Cys. Thus, sulfate assimilation has to be well co-ordinated with assimilation of nitrate and also carbon. Limitations in either nutrient will not only limit the ability to synthesise Cys and Met, but will also limit protein synthesis. Cys formation is the result of succeeding steps starting with sulfate uptake, activation and finally the reduction to sulfide which is then transferred to O-acetylserine. Alterations to any of these three processes can have profound effects on Cys biosynthesis and subsequently on Met levels. Furthermore, the capacity of plants to grow in soils in which nutrient resources are limiting is restricted.

Here, we summarize our current understanding of the regulation of Cys and Met homeostasis in plants, focussing on efforts to understand and manipulate the carbon flux into Cys and Met.

Transcriptome and metabolome analysis of plant sulfur metabolism

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Nutrient ion assimilation needs to be tightly controlled at the biochemical level and to be interlinked with the network of plant metabolism. As availability and distribution of nutrient ions is neither in space nor in time predictable for immobile plants we have to assume flexible adaptation mechanisms. Sulfate is one of the major plant nutrients – next to nitrate/ammonium and phosphate. We challenged *Arabidopsis thaliana* with varying concentrations of sulfate at their rhizosphere. The mechanisms of sensing external nutrient availability and internal nutrient status are still largely unknown. We assume that a part of the responses upon sensing alterations in nutrient availability are to be expected at the level of gene regulation. Thus, we compared the transcriptome of plants under sulfur depleted in comparison to sulfur sufficient conditions. The ultimate effect of the physiological response processes, though, have to be expected as shifts in metabolite fluxes, which at least in part will manifest in alterations of metabolite levels which we scored through a metabolite profiling applying various analytical tools. Depending on the duration of the sulfate nutrient stress hundreds of changes at the transcript level and dozens of detected changes at the metabolite level can be e.g. projected on known pathways to visualize the network response and draw conclusions on interlinkage of respective pathways. Yet, in an attempt to identify novel elements or key regulators in the response of the system we applied bioinformatic tools such as clustering and mutual information content analysis on a fused transcript – metabolite dataset. We propose that such an analysis helps to identify relevant key elements. The analysis of these elements will put forth new knowledge on the regulation of sulfate metabolism in plants.

Transgenic corn with an improved amino acid composition

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Nutritional balance of corn based animal feed requires supplementation with selected amino acids. Our objectives are to reduce or eliminate the need for amino acid supplementation and reduce nitrogen waste in manure by developing corn with an improved amino acid composition. To achieve our goals we have created transgenic corn with substantially increased essential amino acid content in the seed by 1) expressing engineered plant proteins rich in these amino acids and 2) by modification of lysine biosynthetic pathways.

Ectopic expression of foreign polypeptides to high levels requires the ability of the polypeptide to fold and be targeted as efficiently as endogenous proteins and to escape cellular quality control mechanisms in the transgenic tissues. Among a number of candidate genes evaluated, genes encoding an engineered hordothionin and engineered barley high lysine proteins (BHL) resulted in the highest increase of seed lysine. BHL expressing seed also accumulated significantly increased levels of tryptophane and of other essential amino acids. Ectopically expressed engineered proteins accumulated to up to 10% of the total seed protein in multiple independent events.

The aspartate-derived amino acid biosynthetic pathway leads to the synthesis of lysine, methionine, threonine and isoleucine. Activities of several key enzymes in this pathway have been altered to increase lysine and threonine. Aspartate kinase is the first enzyme involved in this pathway. A lysine-feedback insensitive maize aspartate kinase (mAK) was created by site-directed mutagenesis and a feedback insensitive dihydrodipicolinate synthase (dapA) was obtained from Corynebacterium. Transgenic expression of mAK either in the embryo or in the endosperm significantly increased free threonine levels and transgenic expression of deregulated dapA in corn embryos resulted in very high free lysine levels. Expression of neither, dapA or AK in endosperm increased free lysine. Over accumulation of lysine catabolites was observed suggesting that the level of free lysine was controlled by breakdown as well as synthesis. To prevent lysine catabolism, we suppressed expression of the native gene encoding lysil ketoglutarate reductase (LKR) in the endosperm. Corre-

sponding seed showed a moderate increase in lysine and, compared to dapA transformants, reduced saccharopine levels. Transgenic plants overexpressing dapA, mAK or suppressing LKR were crossed. The obtained results reveal a complex relationship between the various enzymes within the aspartate pathway. Plants ectopically expressing high lysine proteins and dapA were also crossed. These plants showed independent and additive trait phenotypes, resulting in very high levels of total lysine (>0.7% of seed dry weight).

Characterization of the mushroom lectin from *Paecilomyces japonica*

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Carbohydrate-binding proteins have been isolated from various sources, including plants, animals, fungi, and bacteria, and they have been used extensively in the detection, localization, and isolation of glycoconjugates. Many carbohydrate-binding proteins are purified from mushrooms, however, only a few proteins with sialic acid-binding specificity have been reported. In the present study, a novel sialic acid-binding protein, designated PJA, has been purified from the mushroom *Paecilomyces japonica*, followed by extraction and affinity chromatography. PJA exhibits hemagglutination activity to human ABO, mouse, rat, and rabbit erythrocytes. This hemagglutination activity is specifically inhibited by *N*-acetylneuraminic acid as well as by glycoproteins containing *N*-acetylneuraminic acid. The carbohydrate-binding activity of PJA was stable at pH values of 4.0–8.0, and at temperatures below 55γØC. The results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis, gel filtration chromatography, and carbohydrate analysis indicate that PJA is a monomer glycoprotein with a molecular mass of approximately 16 kDa comprising a hybrid-type oligosaccharide containing *N*-acetylneuraminic acid, D-mannose, and N-acetyl-D-glucosamine. Although the physiological roles of most reported lectins are yet to be established, we found that PJA exerts cytotoxic effects on human pancreas cancer AsPC-1cells and human stomach cancer SNU-1 cells.

Sialic acid-binding protein from the bark of legume *Maackia fauriei*

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Even though more than 90 lectins have been extracted from legumes, few of them bind specifically to sialic acid derivatives. In the present study, a sialic acid-binding protein has been purified from the bark of legume *Maackia fauriei* using fetuin-affinity chromatography. The eluted fractions from affinity column exhibited hemagglutination activity, thus they are designated as *M. fauriei* agglutinin (MFA). A typical purification method could produce at least 25.5 mg of pure MFA with a specific activity of 40.2 and a recovery yield of no less than 4.9%. A single N-terminal 20-amino acid sequence of MFA, SDELSFNINNFVPN QADLLF, was determined on an automatic Edman degradation amino acid sequencer, and it exhibits a high homology with lectins from *M. amurensis* which binds to sialylated oligosaccharide Neu5Acα2-3Galβ1–3(Neu5Acα2–6)GalNAc. The hemagglutination activity of MFA with human erythrocytes was specifically inhibited by sialylated trisaccharide Neu5Acα2–3Galβ1–4GlcNAc, but it was not inhibited by either Neu5Acα2–6Galβ1–4GlcNAc or Neu5Acα2–3Galβ1–4Glc/Neu5Acα2–6Galβ1–4Glc. This activity of MFA was dependent on divalent cations. Many plant lectins are cytotoxic against cancer cells, with the effect differing with cell type; however, the mechanisms of action remain poorly understood. MFA exerts cytotoxic effects on human breast cancer MCF-7 cells, human melanoma G-361 cells, and human liver cancer SNU-449 cell lines but had no effect on the human colorectal cancer SNU-C1 cell line. It is especially noteworthy that the deleterious effect of MFA on the viability of MCF-7 was greater than that of MAH or wheat germ agglutinin.

Tomato introgression lines as tools to identify regulatory steps of methionine synthesis

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Methionine, an important essential amino acid, is underrepresented in most crop species with respect to human and animal needs. Methionine receives its carbon skeleton from aspartate while its sulphur moiety derives from cysteine. Both pathways for cysteine and methionine synthesis are subject to a complex regulation. In plants the branch point of methionine and threonine synthesis is O-phosphohomoserine (OPHS), which represent the common substrate for both threonine synthase (TS) and cystathionine gamma-synthase (CgS), respectively. With respect to the CgS/TS branch point, overexpression of a feedback insensitive TS in tobacco plants (Muhitch, 1997) resulted in a 5-fold increase in threonine, however no effect in methione level was observed. On the other hand, reducing of the endogenous TS activity in *Arabidopsis thaliana* (Bartlem et al., 2000) and potato leads to in decrease of threonine while methionine levels were dramatically increased by 60- to 200-fold, respectively (Zeh et al., 2001). Surprisingly, the accumulation of methionine was highly variable and developmentally regulated, as methionine contents dropped upon flowering in Arabidopsis. CgS also plays a significant regulatory role in methionine biosynthesis in plants. A mutation in the N – terminal region of the Arabidopsis CgS led to a significant increase in the methionine biosynthesis which led to, a novel hypothesis on post-transcriptional and post-translational regulation of methionine synthesis in Arabidopsis (Amir and Galili, 2002; Chiba et al., 1999; Inaba et al., 1994). The biochemical control of cysteine and methionine biosynthesis is partially understood, a major lack of knowledge occurs concerning signal perception and signalling cascades, which control cysteine and methionine homeostasis. The introgression lines (Zamir) provide a unique resource to obtain novel insights into amino acid biosynthesis in plants, especially in tomato fruits. Through the analysis of these introgression lines it was possible to identify plants with altered methionine contents in fruits. Results will be discussed.

Stimulation of ROS detoxifying enzymes and glutathione metabolism in plants by exogenous 2-aminoethanol

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Exogenous 2-aminoethanol (AE) improves the plant resistance to drought, salinity, chilling injury and some pathogens. Because these stressors induce in plant cells the formation of reactive oxygen species (ROS) the objective in this work is the examination of the influence of AE on the ROS status.

In our experiments with growing barley plants (*Hordeum vulgare* L.) a definable oxidative stress was produced by treatments with the herbizide

paraquat (methyl viologen, 0.1–1 mM). Paraquat promotes the transfer of electrons (from photosystem I) to O_2 strongly.

AE-pre treatments (0.5 mg/plant) reduced the paraquat-induced lipid peroxidation, measured by malondialdehyde. MDA concentration decreased about 25% by AE. And, simultaneously AE increased the SOD activity. (Zymograms in native PAGE.) Paraquat significantly inhibited catalase (CAT) and guaiacol peroxidase (POD) activities. When AE-pretreated plants were exposed to paraquat the CAT-activity was significantly stimulated (without AE: 50 units/g protein, with AE: 90 units/g protein, mean of 0.1 and 1 mM paraquat). The POD activity increased about 36% at 0.1 mM paraquat stress and 20% at 1 mM paraquat stress under the pre-condition of AE treatment.

Paraquat exposure of plants without AE-treatment resulted in an enhanced content of glutathion (GSH increase about 22–34%, 0.1 mM and 1 mM paraquat). In AE-pretreated plants the GSH-content increased 2-fold in relation to untreated plants.

The glutathione reductase (GR) activity decreased by paraquat application. AE could not alter the reduced GR-activity after paraquat treatments. In contrast to this, AE stimulated the glutathione-S-transferase activity in particular after treatments with 0.1 and 1 M paraquat about 75% resp. 30%.

The influence of resistance activation on food and protein production, food quality and food safety was verified.

Transgenic tobacco plants overexpressing the *met25* gene of *saccharomyces cerevisiae* exhibit enhanced levels of cysteine and glutathione and increased tolerance to oxidative stress

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The yeast enzyme *O*-acetylhomoserine sulfhydrylase (AHS), encoded by the *MET25* gene, has been shown by *in vitro* studies also to possess the activity of *O*-acetyl (thiol) lyase (OASTL), the main enzyme in the cysteine biosynthesis, although it does not catalyze this reaction in yeast. In this study, we generated transgenic tobacco plants expressing the yeast *MET25* gene under the control of a constitutive promoter and targeted the yeast protein to the cytosol or to the chloroplasts. Both sets of transgenic plants were taller and greener than wild-type plants. The level of cysteine increased up to four-fold while that of glutathione increased about two and a half fold relative to control wild type plants. The leaf discs of the transgenic plants were more tolerant to toxic levels of sulphite, the substrate of the yeast enzyme, and to paraquat, an herbicide generating active oxygen species. The levels of thiols in seeds of these plants were significantly higher than seeds of wild type plants, and they were more tolerant to droughts conditions. These results demonstrate the potential of the yeast enzyme to enhance the level of cysteine and glutathione in transgenic plants and thereby increase their tolerance to oxidative stress.

Proteomic analysis of storage proteins of maize endosperm mutants

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Maize seeds are a major source of proteins for human and livestock consumption, but the storage proteins are poor in some essential amino acids, mainly lysine and tryptophan. However, maize o*paque* and *floury* mutants have an increased content of lysine in the composition of seed protein. The objective of this study was to compare the protein profiles of the fractions albumin and globulin of the maize endosperms from opaque and floury mutants, *o1*, *o2*, *fl1*, and *fl2*, respectively, with that from the wild-type oh43+. After extraction, protein quantities were determined with the Bradford method, and 50 μg of each fraction was analyzed *via* bidimensional gel electrophoresis (2D-PAGE 8–18% gradient gel) followed by silver nitrate staining. Results showed a similar profile for albumin and globulin from all mutants when compared to that from oh43+. Proteins were distributed between 10 to 120 kDa and isoelectric points (p*I*) between 4 and 9. Analysis of the protein spots through Melanie program version 3 (GeneBio, Sw) reveled absence, presence and differentially expressed proteins of the albumin and globulin fractions from mutants when compared to those from 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.

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Influence of a thermic treatment on the D-amino acid content of corn

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Thermic treatments e.g. extrusion has gained an increasing role in the production of both food and feed products due to its advantageous properties, that is structure formation, inactivation of heat sensitive antinutritive factors, improvement of microbiological quality and increase of digestibility of starch. Heat treatment promotes racemization of free and protein-bonded amino acids, which can result in the decrease of the bioavailability of the amino acids and consumption of the D-amino acid containing food may lead to negative health consequences. Therefore optimalization of the processing conditions is required in order to reach the aim of the process with minimal formation of the D-amino acids. The purpose of the research was to investigate the influence of the heat effect (dependent on the temperature and the residence time applied) on the D-amino acid content of corn grain during extrusion.

In case of corn grain extruded below 140°C with residence time less than 1.25 min (Brabender DC 2001 type machine) the amount of D-amino acids remained less than 1% and did not differ from control. When consuming 200 gramm of these extrudates, the intake of D-amino acids related to 60 kg bodyweight would be no more than 0.35 mg/kg BW for D-aspartic acid and 0.07 mg/kg BW for D-serine. As the result of the treatments at 200°C, among amino acids the racemization of aspartic acid was the most emphasized. Although the increase of D-amino acid content of serine, glutamic acid and leucine was also significant ($P < 0.05$), their increments were far less than that of aspartic acid. This high temperature process resulted in proteins containing 6% of aspartic acid in the form of the D-enantiomer. Consumption of one portion (200 g) of these extrudates involve the intake of 2.1 mg/kg BW D-aspartic acid, and 0.1 mg/kg BW D-serine. However, the amounts of these do still remain significantly lower than levels of *free* D-amino acids applied by other workers in toxicological studies without any pathological changes. Due to extrusion at 200°C, the total (L + D) lysine content decreased with 24% and aspartic acid with 2%. In case of aspartic acid about three-quarters of the loss of the L-enantiomer can be assigned to the D-amino acid formation. Contrast racemization probably is not the primary cause of the loss of L-lysin. Most probably, crosslink formation and side-chain alteration made products of lysine undetectable for amino acid analysis.

Polyamines and Transglutaminases

Direct immobilization of amine oxidases on ethylene-carbon monooxide copolymer

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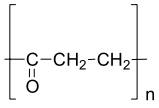
The palladium-catalyzed copolymerization of ethylene and carbon monoxide produces a perfectly alternating polyketone of relatively high crystallinity, to which can be attributed the following formula:



Depending on the polymerization procedure, the ethylene-carbon monoxide copolymer presents some affinity with water. Furthermore, it shows the ability to strongly bind proteins by hydrogen bonds between its keto groups and the amine groups of the amino acids residues of the protein surface and by dipolar interactions. No additional coupling agents or spacer arms are necessary for the immobilization, which is carried out gently, simply mixing end over end the slurry with the protein in diluted neutral phosphate buffer.

The resin was tested with copper amine oxidases isolated from lentil seedling (LSAO) and bovine serum (BSAO) (E.C. 1.4.3.6). In the first case 60 units of enzyme (corresponding to 0.5 mg) have been linked to 100 mg of polymer, giving an apparent LSAO concentration of 1.9 μM in 1.5 ml of slurry. Phosphate buffer 1 M, pH 7.0, containing 1 M NaCl does not remove the protein from the matrix. The immobilized enzyme retains the activity and presents a Km value similar to that shown by the free one. Also specific activity maximum values as a function of temperature and pH are conserved, such as the characteristic biphasic temperature transition.

In the second case, by progressive additions of enzyme, a slurry characterized by an apparent BSAO concentration of 3.6 μM was obtained, without saturating the linking sites of the copolymer.

In conclusion, the matrix appears suitable for enzyme immobilization and purification and for biomedical applications. Biomaterials are important for the controlled delivery of biologically active substances in therapy.

Improvement of bovine serum amine oxidase – spermine toxicity to multidrug-resistant cells by the polyamine oxidase inactivator MDL 72527

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The development of drug resistant tumor cells due to exposure to cytotoxic drugs is one of the major obstacles to successful anticancer chemotherapy. Multidrug resistance (MDR) is associated with several phenotypic alterations. Cells with the MDR phenotype show, among others, a decrease in drug accumulation due to overexpression of P-glycoprotein (P-gp), which acts as an energy dependent pump involved in extrusion of drugs. The purpose of this work was to develop a new strategy to overcome MDR of human cancer cells. By reacting with spermine bovine serum amine oxidase (BSAO) generates cytotoxic products (H_2O_2 and aldehydes). It was shown that cytotoxicity of BSAO/spermine was enhanced by pre-treatment with the polyamine oxidase inhibitor MDL 72527, a compound that had previously been demonstrated to improve the antitumor effect of difluoromethylornithine.

Cell survival experiments were performed on human adenocarcinoma and melanoma cells. These were pre-treated with MDL 72527 at 300 μM, for 24 or 48 hours, and were then exposed to BSAO and 6 μM spermine at 37°C. Cytotoxicity, particularly to MDR cell lines, was significantly higher by the combined treatment, than by BSAO/ spermine alone, even though MDL 72527 did not reduce the number of viable cells under the experimental conditions. An impairment of cell metabolism by this drug was, however, indicated by the formation of numerous vacuoles during the first 24 h of exposure. Their number decreased by 48 h.

The combination of the polyamine oxidase inactivator and the enzymatically formed cytotoxic agents should be of interest in the treatment of multidrug resistant cancers.

The covalent modification of cell proteins by polyamines in cancer prevention

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The elucidation of the role of polyamines remains a challenging problem. Their involvement in the control of events as important as cell proliferation and differentiation is intriguing. Although a simple mechanism of action seems difficult to reconcile with the multiplicity of effects, recent investigations have narrowed the previously proposed hypotheses to a few possibilities. One of the most plausible among these is the regulation of the function of at least some proteins by the posttranslational modification of their structures. In this sense, the specific structural requirements for the activity of polyamines in various organs and cell cultures as modifiers of proteins imply that a common fundamental mechanism underlies their effects. There is considerable evidence to suggest that polyamines may be conjugated to proteins. The studies on the levels of conjugated polyamines in cells have shown a direct correlation between the formation of these compounds and the levels of transglutaminase (TGase) activity. We have been particularly interested in natural agents usually present in the human diet, that suppress cellular growth and induce differentiation involving the posttranslational modification of proteins by structural elements of polyamines. Results indicate that either RA or theophylline induce change in the rate of post-translational modification of protein by polyamines, likely because these drugs increase the activity of TGase and affect the intracellular levels of cAMP. While these data are for the moment based on one histological class of tumor (melanomas), the most important implication of our observation is that it establishes a new way in the screening systems for detecting new molecules able to prevent neoplastic growth.

Ligands as regulators of activity and of stability of transglutaminases

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The activity of tissue tranglutaminase is controlled at the transcriptional level by enzyme induction and at the protein level allosterically by

availability in the cytosol of calcium and of GTP, which respectively activate and inhibit the transmidating protein-crosslinking reaction. *In vitro* investigations have elucidated the molecular basis of the ligand-induced regulatory effects, demonstrating that the reaction is virtually irreversible and is latent at low calcium concentration by the prevailing inhibitory effects of the nucleotide. *In situ* studies have confirmed this regulatory patterns on cultured cells, but also demonstrated that the regulatory ligands also affect tissue turnover of the enzyme, with destabilizing and protective actions by calcium and by GTP respectively, introducing an additional regulatory level involving biodegradation of the protein by cell proteinases. Activation and enzyme degradation are both stimulated by calcium ions and it might be conceived that upon activation the enzyme is rapidly cleared by proteinases, thus limiting unfavourable undue damage to cell structures.

In the present report we demonstrate that this effect of regulated enzyme breakdown applies not only to proteolytic breakdown but also to denaturation by physical (thermal) and chemical (choatropic agents) challenge and is thus related to variations in the intrinsic stability of the protein. This is particularly interesting for tissues, like the kidney, which accumulate large concentrations of denaturating agents like urea (in physiologic conditions) or methylguanidine and guanidinesuccinic acid (in renal failure).

Cobalamin effects on diamine and polyamine oxidase activities in rat liver tissue

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Aliphatic polyamines, spermine and spermidine, as well as their diamine precursor, putrescine, are ubiquitous normal cell constituents that play an essential role in the cell physiology. The principal catabolic pathway of polyamines involves oxidation by polyamine oxidase (EC 1.5.3) to putrescine and 3-aminopropionaldehyde. Putrescine is substrate for diamine oxidase (EC 1.4.3.6). Cobalamin or vitamin B_{12} participates in intermediary metabolism in the form of methylcobalamin and desoxyadenosylcobalamin, in two enzymes in eucariotic cells: methionine synthase and L-methylmalonyl-CoA mutase. Methionine synthase catalyzes the recycling of homocysteine to methionine, using 5-methyltetrahydrofolate as a substrate. Methionine in its active form as S-adenosylmethionine serves as a substrate for the synthesis of polyamines, spermidine and spermine.

From that reason we have examined the polyamine metabolism in liver tissue of rats treated with vitamin B_{12}. Experiment was done with male albino Wistar rats, weighing 250–280 g. The animals received cobalamin in a daily dose of 100 μg per animal during 7 days. Polyamine oxidase and diamine oxidase activities were measured using colorimetric method, based on measuring the amount of formed aminoaldehyde according to the method of Buchrach and Reches, modified by Quash et al. We used putrescine dihydrochloride for diamine oxidase as a substrate.

The results obtained in our experiment point out the increase of polyamine and diamine oxidase activity under the influence of vitamin B_{12}. These results may be explained by the fact that cobalamin augments the amount of polyamines in liver tissue, which potentiates the significance of cobalamin in metabolic pathways of polyamines according to known role of folic acid.

Reactive carbonyl compounds related uremic toxicity ("carbonyl stress")

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Oxidative stress is implicated in the pathogenesis of numerous disease processes including diabetes mellitus, atherosclerosis, ischaemia reperfusion injury and renal failure. Chemical modification of amino acids in protein during lipid peroxidation results in the formation of lipoxidation products which may serve as indicators of oxidative stress *in vivo*. The focus of the studies described here was initially to identify chemical modifications of protein derived exclusively from lipids in order to assess the role of lipid peroxidative damage in the pathogenesis of disease.

Carbonyl stress is characterized as an increase of reactive carbonyl compounds caused by their increased formation and/or decreased breakdown and excretion. The kidney plays a key role in disposal of AGEs, particularly AGE-peptides. We assumed that even a short time combination of enhanced oxidative/carbonyl stress and a lack of renal function should result in elevation of circulating AGE levels. Uremia may be described as a state of carbonyl overload or "carbonyl stress" resulting from either increased oxidation of carbohydrates and lipids (oxidative stress) or inadequate detoxification or inactivation of reactive carbonyl compounds derived from both carbohydrates and lipids by oxidative and nonoxidative chemistry. We propose carbonyl stress as a new uremic toxicity. All experiments were performed on Sprague Dawley rats. Ischemia was induced by 30 min. ligation of *a. renalis* with 24 hours of reperfusion. Myoglobinuric model of ARF was induced by i.m. injection of 50% glycerol. MDA content (8.36 ± 1.48 nmol/mg protein) as well as content of CRD (9.90 ± 1.59 nmol/mg protein) were significantly elevated in kidney in model of ischemia-reperfusion ARF compared to control values (5.33 ± 0.77, $p < 0.01$; 7.85 ± 0.25, $p < 0.01$). Both flavonoids significantly decreased concentration of MDA ($p < 0.001$) and concentration of AGEs ($p < 0.05$) compared to the ischemia/reperfusion group. In myoglobinuric model of ARF in kidney both MDA and AGFs were elevated ($p < 0.01$). Evidence was given that circulating AGEs in the model of acute renal failure in rats undergo a substantial rise within a short time period. A source of increased AGEs is not clear, since except for the lack of the kidney function, accelerated synthesis of AGEs under enhanced oxidative/carbonyl stress as well as liberation of AGEs from tissues due to protein catabolism might be anticipated. If AGEs accumulate in acute renal failure in humans, their contribution to acute toxicity, or of the development of the complications later, might be of importance.

In vitro inhibition of melanoma cell proliferation by plant flavonoids

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Flavonoids, secondary metabolites widely distributed in plant kingdom, are involved in several physiological processes, including plant growth and responses to biotic and abiotic stress. These compounds, as dietary constituents, show a variety of biological effects including some protective activity in the development of degenerative diseases, such as atherosclerosis or cancer. Although dietary polyphenols are considered anti-carcinogens because of their antioxidant activity, direct evidence in support of this relationship is almost lacking, in particular for highly metastatic tumors.

This work has been undertaken to study berries cell suspension cultures for anthocyanins formation as a tool for the production of naturally occurring flavonoids, and to compare the antiproliferative effect of the extracted pigments with pure compounds on highly metastatic B16-F10 melanoma murine cells. Berries cell suspension cultures were obtained from leaf calli of strawberry (*Fragaria* × *ananassa* Duch.), raspberry (*Rubus idaeus* L.) and blackberry (*Rubus fruticosus* L.). To enhance anthocyanin production, growth conditions have been changed, such as type of light exposure (blue light or red light) or temperature (4–25°C). The amount of anthocyanins extracted from control and treated cultures was determined spectrophotometrically. In strawberry cultures, pigment yield was enhanced, after low temperature incubation, by 70%, compared

to control, whereas in blackberry cultures it was increased after red light exposure, by 21%. The higher inhibition of B16-F10 melanoma cell growth, with respect to control, was obtained after treatment of tumor cells with anthocyanin extracts from berries cultures exposed to blue light (blackberry and raspberry extracts: 45% reduction after 72 h). Similar antiproliferative activity was obtained using pure flavonol compounds, naringenin or hesperitin (55% reduction after 72 h). Interestingly, flavonoid-treated cells showed enhanced transglutaminase activity, a well known differentiation marker (65% increase, compared to control).

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Polyamine metabolism in sunflower plants under cd or cu stress

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During the last years, it has been observed a significant increase of metal soil contamination. The aim of this work was to study polyamine metabolism under Cd or Cu stress in sunflower plants exposed to 0.1 and 1 mM of the metals during 16 days. Growth was evaluated measuring the length of roots and shoots and the relative water content (RWC). Both metals produced an inhibition of the shoot growth only at 1 mM. Root growth was reduced by 0.1 and 1 mM Cd or 1 mM Cu. RWC was affected by both metals only at 1 mM from the 7 day of culture (to 60% and 45% of the controls). In shoots, Cd and Cu increased Put content 260% and 160% over the controls, respectively. In roots, both metals inceased Put content from day 7. Spermidine was only increased by 1 mM Cd in both shoots and roots. Spermine content raised in shoots by day 10 under Cd 1 mM and at day 16 with 1 mM Cu. In roots, spermine increased 200% with 1 mM Cd. Regarding biosynthetic enzymes, 1 mM Cu increased ADC activity from day 7, both in shoots and roots, without affecting ODC activity. Cadmium did not modify ADC activity, but increased ODC activity from the third day of treatment. DAO activity was modified differently depending on the day of treatment and/or tissue. The results obtained indicated that polyamine metabolism is modified under Cd or Cu stress, leading to the activation of ADC or ODC biosynthetic routes depending on the metal and tissue.

Enhancement of frameshift by polyamines during translation of prokaryote polypeptide release factor 2

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Prokaryotic polypeptide release factor 2 (RF2) is known to be synthesized by +1 frameshift at the 26th UGA codon of RF2 mRNA. Effects of polyamines on +1 frameshift of RF2 synthesis were examined using *in vivo* and *in vitro* assay systems. Polyamines were found to stimulate +1 frameshift of RF2 synthesis, and excess amounts of RF2 caused to decrease polyamine stimulation of the +1 frameshift. Thus, polyamine stimulation of +1 frameshift of RF2 synthesis was only observed at the early logarithmic phase of cell growth. Shine-Dalgarno (SD) like sequence was necessary for an efficient +1 frameshift of RF2 synthesis, and the codon at the frameshifting site of the mRNA did not influence the polyamine stimulation of the +1 frameshift. The +1 frameshift of antizyme (AZ) synthesis was also stimulated by polyamines in an *Escherichia coli* cell-free system, although SD like sequence did not exist in the AZ mRNA. These results suggest that polyamine enhancement of the +1 frameshift occurs by the structural change of ribosomes by polyamines rather than the structural change of mRNA. Streptomycin, neomycin and tetracycline, but not edeine and pactamycin, caused to decrease polyamine stimulation of +1 frameshift of RF2 synthesis. The results suggest that structural change of A site on 30 S ribosomal subunits by polyamines are important for polyamine stimulation of the +1 frameshift. The −1 frameshift of PolIIIγ synthesis was not influenced by polyamines. Thus, it is concluded that *prfB* encoding RF2 is a new member of polyamine modulon.

Transglutaminase expression in a model of macrophage/microglia activation

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Abnormal increase in transglutaminase reaction may be associated to a variety of pathological conditions, such as neurodegenerative and autoimmune diseases. TGase 2 is increased in autoimmune diseases as a result of macrophage activation, followed by a closely related autoantibody formation. However, it is not clear whether crosslinked inclusion itself is pathogenic. TGase 2 expression in rat brain astrocytes is induced by glutamate or by the inflammation-associated cytokines such as interleukin-1β and TNF-α. Indeed, Parkinson's disease and Alzheimer's disease, are closely associated with increased brain TGase 2 expression, and high levels of inflammatory markers. Considering that a clear evidence indicate that in the injured nervous system, microglia/macrophages are needed at an early stage after injury, we evaluated whether the expression of TGase2 and other TGase isoforms in inflammatory cells in response to various stimuli (TNF-α, IL-6, LPS). We also investigate the possible involvement of NF-κB pathway in TGase modifications. For this purpose, we used the monocytic cell line THP-1, widely used as a model of human monocytes/macrophages or microglia, because of its functional and morphological similarities, including its capacity to perform signal transduction pathways. Our results also demonstrated that different TGase isoforms are expressed in THP-1 cells. The relative expression did not change after inflammatory stimulus, while it did in cells undergoing TPA-induced differentiation to macrophages. As demonstrated by inhibiting NF-κB nuclear translocation with SN-50, the activation of NF-κB pathway can be associated with TGase changes, suggesting an involvement of NF-κB in the inflammation-induced up-regulation of this TGase isoform.

Amine oxidases and reactive oxygen species

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According to the literature, the products of amine oxidases (AOs) mediated oxidation by oxygen of biogenic amines are cytotoxic, being involved in the inhibition of cell growth and differentiation. Cytotoxicity is essentially related to hydrogen peroxide and aldehydes generated in the process. Hydrogen peroxide, which in turn gives various ROS, appears to be cytotoxic in the first minutes of cells incubation with biogenic amines and AOs, at difference from aldehydes, which have to accumulate.

These effects on one hand may be exploited for the inhibition of tumor growth, but, on the other hand, may induce serious damage in important organs and carcinogenesis.

A completely different property of AOs is their antioxidant effect, as demonstrated by the cardioprotection "*ex vivo*" on isolated rat hearts exposed to oxygen free radicals generated by electrolysis. A reduction of ischemia-reperfusion injury "*in vivo*" by pea seedling AO was also demonstrated in rats. More than one mechanism essentially involving histamine oxidation and possibly a ROS scavenger was proposed. In this case AO might prevent the formation of the toxic peroxynitrite by simply removing superoxide before it reacts with nitric oxide forming peroxynitrite.

Bioflavonoid lespeflan and polyamine catabolism in toxic liver damage

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Mechanisms of tissue damage by mercury are not completely clarified, but many studies confirm oxidative stress as important factor in tissue damage. Several studies on humans and animals have shown that consumption or supplementation of food rich in bioflavonoids may have a beneficial effect for human health.

In evaluation of the effect of bioflavonoid Lespeflan on polyamine catabolism we have studied polyamine oxidase (PAO) and diamine oxidase (DAO) in liver tissue after mercury chloride intoxication.

Experiment was performed on male Sprague Dawley rats weighing about 250 g. The animals were killed 24 hours after mercury chloride administration (3 mg/kg). Control group of animals was treated with saline. PAO and DAO activities were measured in the liver homogenate according to the method of Bashrach and Reches (1966). Tissue protein level was determined according to Lowry et al. (1951).

Mercury chloride decreases enzymes activity significantly ($p < 0.05$). Pretreatment by bioflavonoid Lespeflan increases inhibitory effects of mercury chloride on enzyme activities compared to control group ($p < 0.005$).

Results obtained in this study indicate that polyamines have important contribution in mercury chloride toxicity. Bioflavonoid Lespeflan modulates PAO and DAO activities in mercury chloride intoxication

Polyamines as modulators of redox signaling pathways in diabetic rats

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Oxidative stress occurs in diabetic patients and experimental models of diabetes. It was shown that the activation of PKC as well as MAPK by hyperglycemia, ROS and AGE products may affect redox-senzitive signaling pathway of the cell, representing the critical event in the development of vascular complication in diabetics. The ability of polyamine spermidine (Spd) to ameliorate oxidative stress and metabolic changes after treatment with streptozotocine was investigated in the liver and kidney of diabetic rats. Hyperglycaemia, hypertriglyceridemia and increased AST activity were observed in serum after 15 days of streptozotocine (St) treatment. This was associated with a depression of glutathione (GSH) concentration as well as the thiobarbituric acid-reactive substances (TBARS) elevation in the liver and kidney. In addition, the PAO (polyamine oxidase) and DAO (diamine oxidase) activities were significantly elevated, indicating increased polyamine catabolism. Based on these findings as well as on the fact that the concentrations of GSH, TBARS, polyamines and PAO and DAO activities were reversed by simultaneous treatment with St and Spd, and that Spd given alone significantly increased the content of GSH, we postulated that there is a ''cross-talk'' between polyamines and down regulation of redox cell signalization. Thus, these results showed that exogenously administered spermidine might improve the clinical manifestation of diabetes mellitus and decrease the oxidative stress in the liver and kidney.

Dissecting TG2 function *in vivo*

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Transglutaminases (TGs) are a family of calcium dependent cross-linking enzymes involved in post-translational modification of proteins. They catalyze intra- and inter-molecular iso-dipeptide bonds between the gamma-carboxyamide group of glutamine residues and the primary amino groups of several compounds. The most ubiquitous isoenzyme of the family is ''tissue'' or ''type 2'' transglutaminase (TG2), a versatile multifunctional protein involved in a variety of biochemical functions. High $Ca2+$ levels activates TG2 to catalyze protein–protein cross-linking, incorporation of primary amine into protein as well as glutamine deamidation. TG2 has been shown to play a pathogenetic role in Alzheimer's and Huntington's disease. To examine whether TG2 plays a role in the nigro-striatal degeneration in an experimental model of parkinsonism, we used TG2 knockout mice challenged with the nigro-striatal toxin 1-methyl–4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). In wild type mice, high doses of MPTP ($20\,mg/kg \times 4$) induced a nearly total degeneration of the nigro-striatal pathway, as shown by measurements of striatal dopamine (DA) levels and by immunohistochemical analysis of tyrosine hydroxylase, high affinity DA transporter, and glial fibrillary acidic protein in the corpus striatum and substantia nigra. Lower cumulative doses of MPTP (30 mg/kg) produced a partial lesion of the nigro-striatal pathway. In TG2 knockout mice, the systemic injection of high and low doses of MPTP induced a significant lower degree of nigro-striatal degeneration. No difference in the central bioavailability and the local half-life of MPTP, as shown by measurements of the toxin and its active metabolite, MPP^+, in the striatum was observed in TG2 knockout mice as compared with wildtype mice.

In another recent study we also demonstrated that, under physiological conditions, TG2 acts as a protein disulfide isomerase (PDI) and this activity plays an important role in the correct assembly of the mitochondrial respiratory chain complexes in the heart. Mice lacking TG2 exhibit impairment of mitochondrial energy production, evidenced by decreased ATP levels after physical challenge, and enhanced sensitivity to *in vivo* inhibition of respiratory chain complex II. The resulting phenotype exhibits a decrease in motor behavior and overall general activity of the animals. The molecular mechanism responsible for such a syndrome rely on the defective formation of disulphide bonds in specific protein subunits of respiratory chains. Our data elucidate a new pathway that directly links the new TG2-PDI enzymatic activity with the mitochondrial respiratory chain biogenesis and function.

We conclude that TG2 plays an important role in Neurodegeneration and its inhibition may be a novel therapeutic target to slow down the progression of neuronal death.

A modeling approach to the study of arginine metabolism

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Mathematical modeling for simulation-based analysis is becoming a key tool for the development of systems biology, the analysis of the relationship among the elements in a system in response to genetic or environmental perturbations, with the goal of understanding the system as a whole. Recently, several interesting and relevant mathematical models of well known metabolic pathways have been published, including one describing the branched chain amino acid biosynthetic pathways of *E. coli*. We are interested in the modeling of arginine metabolism in mammals. The cationic amino acid arginine is the precursor of different biomolecules with very relevant physiological roles, such as urea, polyamines and nitric oxide, among others. Within the framework of arginine metabolism, we focus our interest in the branched pathway leading to either nitric oxide (*via* nitric oxide

synthase) or polyamines (*via* arginase and ornithine decarboxylase). Although the different elements of this system are reasonably well known, our aim is to integrate the available data to explain its dynamics in a formal and predictable way. To fulfil this goal, we make use of a modeling and simulation approach that will be described in the present communication.

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Polyamines metabolism in some Leptospira species infectious or not for human beings

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Introduction: The genus Leptospira comprises a heterogenous group of pathogenic and saprophytic species belonging to the order Spirochaetales. Leptospires are thin, helically coiled, motile bacteria, serologically classified into hundreds pathogenic serovars. The pathogenic and saprophytic leptospires are similar in morphology but differ in several biological characteristics. When compared with the pathogens, the saprophytes manifest greater resistance to copper sulphate and 8-azaguanine, and posses higher oxidase and egg-yolk decomposition activity. The saprophytes produce a mouse-erythrocyte haemolytic factor, and certain types can grow in the absence of CO_2. The saprophytic leptospires are indigenous to fresh surface waters, and neither a parasitic nor a saprophytic role has been firmly established for them. Mammals are not susceptible to experimental infection by these leptospires. Leptospiral pathogenic serovar diversity results from structural heterogeneity in the carbohydrate component of lipopolysaccharides. Many serovar are adapted for specific mammalian reservoir hosts, which harbour the organisms in their renal tubules and shed them in their urine. Because of the large spectrum of animal species that serve as reservoir, leptospirosis is considered to be the most widespread zoonotic disease. Transmission to humans occurs through contact with wild or domestic animals or exposure to contamined soil or water. In the urban setting, L. interrogans serovars that colonize the brown rat population are the predominant cause of disease in humans. Fever, chills, headache, and severe myalgias characterize the early phase of disease. Progression to multiorgan system complications occurs in 5 to 15% of cases, with mortality rates of 5 to 40%. Leptospirosis is also a major economic burden as a cause of disease in livestock and domestic animals. Environmental control measures are difficult to implement, and currently available inactivated whole-cell vaccines have a number of disadvantages. Polyamines (putrescine, spermidine and spermine) are necessary for cell growth. They are among the major polycations in cells, and their content is regulated by biosynthesis, degradation and transport. Putrescine and spermidine are the major polyamines in bacteria, and putrescine can be formed directly from ornithine by ODC in animal and fungal cells. In some bacterial cells is an additional indirect route to putrescine from arginine by ADC. Spermidine synthase synthesises spermidine from putrescine by the addition of an aminopropyl group of decarboxylated S-adenosylmethionine. A furter transfer on spermidine yields spermine in some organisms. In this work we have investigated the polyamines metabolism in some leptospira species to attempt another addition on the differentiation of pathogenic and saprophytic leptospires.

Materials and methods: All leptospira strains, obtained from Parasitological Center of Messina University, were grown to midlogarithmic phase at 30 °C in Korthof leptospiral enrichment medium for at least 1 week before use. Ten millilitre of each culture was washed three times in sterile PBS, centrifuged and the pellet was resuspended in PCA, final concentration 0.2 N, and homogenized. Polyamines in PCA extract were kept on ice and centrifuged at 28,000 × g for 10 min, and supernatants were derivatized and assayed according to Flores and Galston.

Results and discussion: Our results show that DAP and spermidine are the major and in many cases the only polyamines present in all strains of leptospires investigated in this study. When compared with the saprophytes, the pathogens display greaters levels of spermidine and DAP and often non detectable contents of putrescine, that is instead appreciable in saprophytic strains, even if low.

Polyamine dependent kidney growth mediates glomerular hyperfiltration in early diabetes

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Diabetes mellitus results in a multifactorial etiology, yet the mechanisms responsible for the onset and progression of this disease have not been fully resolved. In diabetes, supranormal glomerular filtration rate (GFR), i.e hyperfiltration, and kidney growth are considered primary events, with sclerosis and progression to end-stage renal disease (ESRD) occurring some years later. Unrelenting hyperfiltration damages the glomerulus. This leads to progressive complications such as oxidative stress, increased apoptosis of the proximal tubules, and fibrosis. Although unproven, it has been presumed that diabetic vasodilation results in hyperfiltration, and that hyperfiltration leads to compensatory kidney growth. Numerous vascular mediators have been proposed in support of this model. Here we present evidence of an opposing view, one in which growth is not the result of, but rather the primary mediator of, glomerular hyperfiltration. How does growth impact hyperfiltration? With regard to total volume, growth of the diabetic kidney is attributed primarily to the proximal tubule. In diabetes proximal tubule growth correlates with an abnormal increase in sodium reabsorption. This abnormal reabsorption leaves less salt to be sensed downstream by the macula densa that feedback controls GFR. Thus, low salt at the macula densa, as presented in diabetes, signals the system to increase GFR in order to increase the salt load to the macula densa. Because of abnormal reabsorption upstream at the proximal tubule, this feedback system is dysfunctional and consistently signals for high GFR, and thus hyperfiltration.

In proximal tubular growth, a period of hyperplasia precedes diabetic hypertrophy. BrdU staining, indicative of proliferation, was maximal 3 days after initiation of experimental diabetes with STZ, and completely receded by day 7. DFMO treatment markedly inhibited BrdU staining at day 3 in response to STZ, indicating ODC dependent growth. ODC expression and activity was increased with STZ, and activity inhibited with DFMO treatment. A direct correlation between kidney growth and GFR was demonstrated in STZ diabetes in rats. Importantly, suppressing STZ mediated kidney growth with DFMO suppressed the increase in GFR to a corresponding degree. This demonstrates a direct correlation in growth increasing GFR in diabetes. Increased ODC expression in response to STZ was localized in the distal, not the proximal, tubules. Work is ongoing to sort out this apparent paracrine effect of distal tubule polyamine biosynthesis with proximal tubule growth.

Conclusion: Optimal insulin treatment forms the modern basis for preventing organ damage in diabetes. However, exogenous insulin cannot match the kinetics of a healthy endocrine system. These data identify polyamine dependent kidney growth as direct cause of renal glomerular hyperfiltration. Understanding the role of growth in the pathogenesis of diabetes will offer new opportunities for early diagnosis and therapeutic intervention.

Effect of putrescine on intensity of lipid peroxidation in rat brain with cholestasis

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Encephalopathy in cholestasis results from accumulation of unconjugated bilirubin (UCB) and hydrophobic bile acids (BA). Toxic BA and UCB induces neurotoxicity (apoptosis of neurons). Putrescine, spermidine and spermine are endogenous polyamines essential for cellular growth, regeneration and differentiation. Beneficial effects of putrescine in CNS injury have been attibuted to anti-apoptotic ant anti-oxidant properties.

The aim of the study was to examine the effect of putrescine at the level of lipid peroxidation in cholestatic brain injury.

Wistar rats were divided into 5 groups: I-control, II-sham operated rats, III-treated only with putrescine, IV-bile duct ligated (BDL) rats, V-BDL rats treated with putrescine (150 mg/kgBW ip.). The animals were killed after 9-day treatment.

Administration of putrescine in BDL rats reduces concentration of blood plasma UCB and BA (29.2 ± 3.3 vs. $43.6 \pm 5.9\,\mu$mol/l and 11.4 ± 0.8 vs. $22.8 \pm 2.6\,\mu$mol/l; $p < 0.001$). The lipid peroxidation (MDA) was increased in brain of BDL rats (4.98 ± 0.54 vs. control 3.95 ± 0.32 nmol/mg prot; $p < 0.001$). Putrescine decreased MDA level in brain of V group vs. IV group rats (2.25 ± 0.42 vs. 4.98 ± 0.54 nmol/mg p; $p < 0.001$). The amplification of cerebral activity of polyamine oxidase (PAO) in BDL rats (1.25 ± 0.09 vs. control 0.84 ± 0.09 U/mg prot; $p < 0.001$), resulted in high local concentrations of 3-acetamidopropanol and H_2O_2 which lead to oxidative stress and cell death. Administration of putrescine of BDL rats, decreased activity of cerebral PAO compared with BDL rats (1.02 ± 0.07 vs. 1.25 ± 0.09 U/mg prot; $p < 0.001$).

Administration of putrescine in BDL rats results in normalization of cerebral oxidative stress and has protective role after the CNS injury in cholestasis.

Metabolism of polyamines in rats' brain with extrahepatic cholestasis

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The pathogenesis of encephalopathy in cholestasis results from accumulation of unconjugated bilirubin (UCB) and hydrophobic bile acids (BA) in brain. Toxic BA and UCB induce neurotoxicity and being transported across the blood-brain barrier, they are accumulated at the target neurons. Putrescine, spermidine and spermine are endogenous polyamines essential for cellular growth, proliferation, regeneration and differentiation. The amino-acid L-Methionine (L-Met) is required for the biosynthesis of polyamines.

The aim the study was to examine effect of L-Met in polyamine metabolism on cholestatic brain of injured rats.

Wistar rats were divided into 5 groups: I-control, II-sham operated rats, III-treated only with L-Met, IV-bile duct ligated (BDL) rats, V-BDL rats treated with L-Met (50 mg/kg BW). The animals were killed after 9 days treatment.

Increased plasma cholestatic markers activity (GGT, AF and ALT) in BDL rats was decreased by oral administration of L-Met ($p < 0.001$).

Cholestasis in rats' brain increases the putrescine level (110 ± 13.2 vs. 65 ± 6.8 nmol/g; $p < 0.001$) and decreases spermidine (298 ± 19.2 vs. 318 ± 19.5 nmol/g; $p < 0.05$) and spermine concentration (203 ± 16.2 vs. 225 ± 12.7 nmol/g; $p < 0.05$), in relation to sham operated rats. The increase of putrescine level after CNS trauma is adaptive neuroprotective responses.

The activity of PAO is increased ($1.25 + 0.09$ vs. $0.81 + 0.08$ U/mg prot; $p < 0.001$) and activity DAO is reduced ($0.33 + 0.06$ vs. $0.68 + 0.10$ U/mg prot; $p < 0.001$) in brain of BDL rats compared with sham operated rats. Administration of L-Met in BDL rats prevents disorder of biosynthesis and catabolism polyamines in brain during cholestasis.

L-Met is important for the regulation of polyamines metabolism and demonstration of neuroprotective role in cholestasis.

Polyamine depletion inhibits both the death receptor pathway and the mitochondrial pathway of apoptosis in chondrocytes

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Chondrocytes, the only cell type in mature cartilage, are responsible for the synthesis and metabolic control of the extracellular matrix. Thus, cartilage integrity depends on the biochemical and cellular functions of chondrocytes. Chondrocytes show a limited proliferative activity in response to injury, making chondrocyte survival critical for the preservation of cartilage structure and function. Chondrocyte apoptosis can be an important contributor to cartilage degeneration, thereby making it a potential therapeutic target in articular diseases. Although it is well known that polyamines are essential for cell proliferation, growing evidence also suggests a role for polyamines in apoptosis. Therefore, to search for new approaches to limit chondrocytic cell death, we investigated the requirement of polyamines for apoptosis in human chondrocytes. Apoptosis was induced in chondrocytes by the combined treatment of tumor necrosis factor-α (TNF) plus cycloheximide (CHX) or TNF plus MG-132, to explore the death receptor pathway, or by staurosporine, a well known apoptosis inducer that acts through the mitochondrial pathway. Polyamine depletion was obtained by treatment of chondrocytes with specific polyamine biosynthesis inhibitors, i.e. α-difluoromethylornithine (DFMO), an ornithine decarboxylase inhibitor, or CGP 48664, a S-adenosylmethionine decarboxylase inhibitor. The results suggest that the intracellular depletion of polyamines in chondrocytes can inhibit both the death receptor pathway by reducing the level of procaspase-8, and the apoptotic mitochondrial pathway by activating Akt.

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Polyamine and nitric oxide metabolism during arginine pretreatment in experimentally induced seizures

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Epileptogenesis comprises molecular and cellular events producing the disordered firing of a subpopulation of neurons resulting in periodic seizures. Events observed in both human and animal epilepsies are increase in glutamate levels, NMDA receptor sensitivity, selective loss of pyramidal neurons and neosynaptogenesis. It has been found that prolonged NMDA receptor – dependent epileptiform discharges originate in cortex and propagate to hippocampus *via* perforant path – dentate gyrus route. These changes in network interactions along with other mechanisms of synaptic plasticity can confer to the epileptic, damaged limbic system with the ability to produce recurrent limbic seizures.

The important end-products of L-arginine metabolism are cell-signaling molecules (nitric oxide (NO), glutamate and agmatine), while polyamines, although not commonly thought of as cell-signaling

molecules, can also regulate key regulatory processes, such as ion channel function.

The experiment comprised 7 experimental groups of animals (control, penthylene tetrazol treated and 5 groups treated by arginine in five different doses). The obtained results (clinical findings and biochemical analyses of polyamine and nitric oxide metabolism enzymes, performed in six different brain structures) showed the strong effort of CNS to maintain cell putrescine pool, presumably by the activation of polyamine interconversion pathway, during seizures.

Pretreatment with five different doses of arginine during seizures induced by pentylene tetrazole showed the complexity of the regulation of its metabolic pathways (which is not just the simple competition between arginase and nitric oxide synthase) and the importance of polyamine metabolism changes during seizure activity, confirming these changes as protective mechanisms of CNS in seizures.

Changes in prostate polyamine metabolism by green tea catechins in autochthonous mouse model of prostate cancer (TRAMP mice)

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Chemopreventive action of Green tea catechins (GTCs) was proven effective against cancer in several experimental models of carcinogenesis. Transgenic adenocarcinoma of the mouse prostate (TRAMP) is an excellent model of prostate cancer that mimics progression of human disease from prostate intra-epithelial neoplasia (PIN) to androgen-independent disease with distant site metastases. Putrescine (Put), Spermidine (Spd) and spermine (Spm) are necessary for normal and pathological cell growth and differentiation. In human prostate the levels of expression of all the biosynthetic regulatory genes of polyamine metabolism are significantly higher in the neoplastic tissue than in the benign counterpart. In TRAMP mice prostate, the activity of ornithine decarboxylase (ODC), principal regulatory enzyme of polyamine biosynthesis, and protein level were found markedly higher than in nontransgenic littermates. Other authors have found that overexpression of spermidine/spermine N^1-acetyltransferase (SSAT), the enzyme regulating the catabolism and export of intracellular polyamines, caused a sustained suppression of tumour outgrowth in TRAMP mice. This suggests the suitability of polyamine metabolism as a target for chemopreventive action of GTCs. We treated TRAMP mice with GTCs in drinking water at 0.3% to investigate their effectiveness in restoring normal levels of expression of the regulatory genes of polyamine metabolism. Preliminary results showed that, as expected, ODC and S-adenosylmethionine decarboxylase (AdoMetDC) transcripts increased in 24 weeks old TRAMP mice prostate glands, where cancer is fully developed, in comparison to wild mice of the same age. GTCs treatment was apparently ineffective in reducing ODC mRNA, while being able to decrease AdoMetDC mRNA. SSAT transcript level did not change in 24 week old wild and TRAMP mice. However, GTCs treatment increased SSAT mRNA in 24 weeks TRAMP mice. The transcript for ornithine decarboxylase antizyme (OAZ), the ODC inhibitor protein, decreased during cancer progression in TRAMP mice. This was prevented by GTCs administration. Put and spd concentrations in TRAMP mice were enhanced at 24 weeks as compared to 12 weeks of age, but Spm decrease substantially. The increase of Put and Spd was almost completely prevented by catechin administration.

The data presented here confirm the involvement of polyamines in TRAMP mouse prostate cancer development. It appears that Put and Spd are required for supporting tumour cell proliferation, but Spm is only augmented at the beginning of tumorigenesis (12 weeks), while at 24 weeks, when the tumour is fully developed, Spm markedly decreased, suggesting that growth of highly malignant cells might require lower levels of Spm, a molecule involved in cell differentiation. The effect of GTCs treatment on TRAMP mice shows that the chemopreventive effect of these compounds might, at least in part, be due to their inhibitory action on Put and Spd production

Proteomics

Protein sites of attack of N-chlorotaurine in Escherichia coli

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Introduction: The aim of this pilot study was to get information about the mechanism of action of the new antiseptic NCT, which is a promising microbicidal agent for topical treatment of infections.

Escherichia coli, a species with a well-known protein pattern in proteomics databases, was chosen as a test organism.

Methods: The bacteria were treated with NCT for 10 and 30 min – a period were killing takes place. To find out protein changes of Escherichia coli, 2D-PAGE followed by mass spectrometry was performed.

Results: Incubation in 1% NCT for 10 and 30 min revealed a change of the charge and a separation of numerous proteins into a series of spots with a different isoelectric point.

Heat shock protein 60 appeared, while ribosome releasing factor, D-ribose periplasmic binding protein, and malonyl-CoA transacylase spots decreased. Molecular mechanisms of attack comprised mainly oxidation of thio and amino groups.

Conclusion: These results indicate penetration of oxidation capacity into the bacteria and destruction of essential proteins by NCT.

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Amino-terminal dimerization of the nonapeptide HLA-DR fragment on the solid support

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Dimerization of receptors is an essential step of various cellular signal transduction processes. Substances that are able to modulate the receptors dimerization may control such a process and are of potential therapeutical value. Dimeric ligands are known candidates for mediating dimerization of these types of receptors.

The nonapeptide fragment of HLA-DR, located in the β164–172 loop with the VPRSGEVYT sequence, suppresses the immune responses. It has been suggested that the loop may serve as a functional epitope on the HLA class II surface for intermolecular binding, and that a possible mechanism of biological action of the synthesized peptides is associated with their specific interference in the interaction of HLA class II molecules with their coreceptors. It was postulated that oligomerization of the coreceptors is required for initiation of the immune response. Dimerization of analogs of the immunosuppressive HLA-DR fragment, by connecting their C-termini to two amino groups of lysine, was previously shown to be beneficial in terms of potency and specificity of immunosuppressive action.

Based on three-dimensional structure of the HLA-DR superdimer, we designed the dimeric analogs able to mimic the dimeric nature of the immunosuppressory fragments of HLA class II molecules. Using suitably modified standard solid-phase peptide synthesis protocols, dimeric analogs of the nonapeptide HLA-DR fragment were synthesized. The dimerization was achieved by crosslinking N-terminal positions of peptides attached to an MBHA-resin with poly (ethyleneglycol)bis(carboxymethyl)ether activated by estrification with pentafluorophenol. Our results demonstrated that the N-terminal dimerization of the nonapeptide fragment of HLA-DR resulted in enhanced immunosuppressory properties and the effect depended on the linker length.

Release of amino acid neurotransmitters under the action of short peptides corresponding to the definite domains of nerve proteins GAP-43, BASP1 and NCAM

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Synaptic transmission is the main mode of communication between nerve cells in brain. However, many details of this process are not elucidated yet. In this study, we investigate involvement of growth associated proteins, GAP-43 and BASP1 (CAP-23, NAP-22), as well as neural cell adhesion molecule (NCAM) in neurotransmitter release by rat synaptosomes (isolated axonal endings of neurons) treated with synthetic peptides that correspond to definite domains of the above-mentioned proteins. Neurotransmitters released by both treated and control synaptosomes were analyzed by HPLC and TLC. We have shown that peptides P2 and FGL (recently identified peptide mimetics of NCAM; Berezin and Bock, 2004) efficiently induced release of several amino acids by synaptosomes. In synaptosomes treated with P2 and FGL, the phosphorylation of ''signal'' proteins GAP-43 and BASP1 by PKC is highly stimulated. Therefore, GAP-43 and BASP1 are likely involved in NCAM-induced neurotransmitter release. In other series of experiments, we treated synaptosomes with peptides corresponding to the N-terminal domain of GAP-43 (GAP-43 (1–10)), the effector domain of GAP-43 (GAP-43 ED) and the N-terminal domain of BASP1 (BASP1 (1–12)). It proved that peptides corresponding to the both domains of GAP-43 stimulated neurotransmitter release, while BASP1 (1–12) had no effect. The obtained results demonstrate the usefulness of a relatively simple subcellular system (synaptosomes) for studying signaling mechanisms that control neurotransmitter release. Supported by RFBI(05-04-49142) and INTAS(011-0064) grants.

Reducing protein concentration range of biological samples using combinatorial ligand libraries

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The discovery of polypeptides/proteins of diagnostic relevance from a biological liquid is complicated by their vast number and the large concentration range. Depletion methodologies or fractionation have been used but they failed to significantly enrich trace. Here we report a new that allows the reduction of protein concentration range of a complex mixture like neat serum through the simultaneous dilution of high abundance proteins and the concentration of low abundance proteins in a single, simple step. This methodology utilizes solid-phase ligand libraries of large diversity, generated *via* standard ''split, couple, recombine'' combinatorial chemistry. With a controlled sample-to-ligand ratio it is possible to modulate the relative concentration of proteins such that a large number of peptides or proteins that are normally not detectable by classical analytical methods become easily accessible.

Application of this method to reduce the dynamic range of unfractionated serum is specifically described along with treatment of other biological liquids. Analytical SELDI MS technology and mono- and bi-dimensional electrophoresis demonstrate the reduction in protein concentration range. Specific examples linking this approach with additional fractionation methods demonstrate a further increase of the number of detectable species.

Cell-activation through Toll-like receptors: TLR6 dependence of triacylated lipopeptides

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Lipoproteins are part of the outer membrane of Gram-negative bacteria, Gram-positive bacteria, Rhodopseudomonas viridis, and mycoplasma. They are characterized by the N-terminal unusual amino acid S-(2,3-dihydroxypropyl)-L-cysteine (Dhc) acylated by two or three fatty acids ($Acyl_2Dhc$ or $Acyl_3Dhc$). $Acyl_3Dhc$ contains two ester-bound and one amide-bound fatty acid moiety, $Acyl_2Dhc$ contains only two ester-bound fatty acid moieties and a free N-terminus. Synthetic analogues of the N-terminal part of these lipoproteins constitute potent immunoadjuvants *in vivo* and *in vitro*.

Cell activation by lipoproteins and shorter lipopeptides is induced through their interaction with Toll-like receptor 2 (TLR2). TLR2 forms heterodimers with either TLR1 or TLR6 to attain specificity for a given stimulus. Diacyl lipopeptides like macrophage activating lipopeptide from Mycoplasma salivarium (FSL-1, Pam_2Cys-GDPKHPKSF) or Mycoplasma fermentans (MALP-2, Pam_2Cys-GNNDESNISFKEK) were described to require TLR2 and TLR6 for signalling, whereas triacyl-lipopeptides like Pam_3Cys-SK_4 are able to activate immunocompetent cells independently of TLR6 mainly through TLR2/TLR1 heterodimers. TLR6 appears to confer the ability to discriminate between the N-terminally acylated and not acylated lipopeptides.

A systematic approach was performed to investigate the structural influence of the amide-bound fatty acid moiety of triacylated lipopeptides on the TLR2/TLR6 dependent activation of murine B-lymphocytes. Therefore Pam_2Cys-GNNDESNISFKEK and lipopeptides Ac_3Cys-GNNDESNISFKEK differing in length and character of the amide-bound fatty acid moiety were synthesized and tested for their ability to stimulate TLR6 wild-type and TLR6 deficient murine B-lymphocytes.

Proteomic strategies for monitoring milk protein quality

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Dietary protein quality is dependent on several factors, not only protein and amino acid composition, but also protein digestibility and amino acid bioavailability, the latter being susceptible to major modifications after processing of food.

Rapid development of the proteomic technique and recent application to the study of food protein quality has revealed its potentiality in pointing out differences relevant for nutrition. Advanced electrophoretic techniques (two-dimensional gel electrophoresis, capillary electrophoresis) and matrix-assisted laser desorption ionisation (MALDI) mass spectrometry offer a new approach to study interactions between proteins and other components, or protein–protein interactions, during processing. Proteomic techniques have been shown to represent a powerful tool in the analysis of protein in complex matrix, such as milk and dairy products, suitable to characterization of typical products, too. On the other hand, few studies are available on digestion patterns of food proteins and, therefore, on factors affecting their bioavailability.

The aim of this study was to provide information about strategies of proteome analysis and its application to important food proteins, with special relevance to milk proteins.

Application in protein quality determination was focused on the search of quality markers of a national typical product, buffalo mozzarella from Campania, that obtained protected designation of origin (DOP).

Proteomics was also applied for traceability of casein and whole milk proteins during *in vivo* gastrointestinal digestion in a rat model system. Control of proteolysis was performed to check for the lack of antigenic proteins in novel milk formulas.

The work was supported by the Italian Ministry of Agricultural and Forestry Policies, Project ''QUAGRI''.

Determination of soybean proteins added to commercial heat-processed meat products prepared with chicken, beef and mixtures of meats of different species by perfusion reversed-phase high performance liquid chromatography

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Emulsion type heat-processed meat products are commonly consumed in modern societies. These products can present a complex composition containing meats from different species and even non-meat proteins. Indeed, non-meat proteins such as soybean proteins are added to meat products to prevent coalescence of fat during heating and as fat replacers.

The addition of soybean proteins to meat products is regulated being allowed up to a certain degree or forbidden depending on the type and quality of the product. Despite these regulations, there is not any reliable method enabling the control of these products. This problem has been approached in this work.

Therefore, the determination of additions of soybean proteins in emulsion-type heat-processed meat products prepared with chicken, beef, and mixtures of meats of different species (chicken-pork, beef-turkey, pork-chicken-turkey, beef-turkey-chicken, and pork-beef-chicken-turkey) that could also contain milk proteins has been carried out by perfusion reversed-phase high performance liquid chromatography. The proposed method enabled the detection and quantitation of additions of up 0.08% (w/w) and 0.27% (w/w), respectively, of soybean proteins in meat products and did not suffer from matrix interferences. Validation of the method was successful performed by evaluation of the linearity, specificity, precision and recovery. Moreover, accuracy was evaluated by comparison of soybean protein contents determined by the proposed method and the ELISA AOAC method.

Determination of soybean proteins in soybean-cereal (wheat, corn, and rice) protein complex mixtures by perfusion reversed-phase HPLC. Application to the quantitation of soybean proteins in commercial bakery products

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Cereal grains are main foods in many countries. Despite their interesting nutritional and functional properties, cereal proteins present a deficiency in certain essential amino acids such as lysine. Since soybean proteins constitute a source of high quality proteins and are rich in lysine, its amino acid profile fits nicely with that of cereal proteins. In addition, soybean proteins have other advantages such as low cost and interesting functional properties. The increasing use of soybean proteins in the manufacturing of bakery products has resulted in the establishment of regulations forbidding or limiting the addition of soybean proteins to these products.

Although many works have been devoted to the development of analytical methodologies for the study and characterization of cereal proteins, the simultaneous separation of soybean and cereal proteins has never been approached. Therefore, the aim of this work was to develop an analytical methodology enabling the separation of soybean proteins from cereal (wheat, rice, and corn) proteins in complex mixtures for its application in the determination of soybean proteins in commercial bakery products.

A perfusion HPLC method consisting of a linear binary gradient water-acetonitrile-0.3% acid acetic with a temperature of 50°C and UV detection at 254 nm has been developed. The method was successfully applied to the determination of soybean proteins in commercial bakery products manufactured with binary, ternary, and quaternary soybean-cereal mixtures.

Targeted down regulation of topoisomerase II beta leads to altered response to retinoid induced myeloid differentiation and cell death

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Topoisomerases are essential for altering DNA topology during vital cellular processes. Among the type II topoisomerases, topoisomerase (topo) IIα is primarily involved in cell proliferation, whereas topo IIβ has been suggested to regulate cell differentiation. In this study we examined the functional role of topo IIβ in all-trans retinoic acid (ATRA)-induced differentiation of acute promyelocytic leukemia (APL) cell lines, HL-60 and NB4. The topo IIβ specific inhibitor, ICRF-193, potentiated ATRA-induced differentiation and enhanced cell kill of differentiated HL-60 cells. Down regulation of topo IIβ in HL-60 and NB-4 cells with topo IIβ-specific si-RNAs also led to apoptosis and reduced survival of differentiated cells, without altering ATRA-induced differentiation. Similar results were observed in another topo IIβ deficient HL-60 cell line derived by selection for resistance to the topo

IIβ-targeted drug, amsacrine. In contrast, down regulation of topo IIα with an si-RNA did not alter ATRA-induced differentiation or apoptosis. Reduced expression of topo IIα in HL-60 cells however compromised the cytotoxic effects of the topo IIα-targeting drug, etoposide, but not of the topo IIβ-targeting drugs, amsacrine and mitoxantrone. Analysis and validation of gene expression profiles in topo IIβ expressing (si-GFP) and topo IIβ deficient (si-topo IIβ) cells revealed significant down-regulation of peroxiredoxin 2 (PRDX2) in si-topo IIβ cells, and induction of regulator of G-protein signaling (RGS2) in ATRA-differentiated si-topo IIβ cells. These results demonstrate that topo IIβ, but not topo IIα, is required for maintaining survival of ATRA-differentiated APL cells probably *via* a mechanism involving down-regulation of PRDX2 and up-regulation of RGS2.

Differentially expressed proteins by *Helicobacter pylori* infection in human gastric epithelial cells

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Helicobacter pylori infection leads to gastroduodenal inflammation, peptic ulceration, and gastric carcinoma. Proteomic analysis for human gastric epithelial cells, which were either infected or non-infected with *Helicobacter pylori*, was used to determine the differentially expressed proteins by *Helicobacter pylori* in human gastric epithelial cells to investigate the pathogenic mechanism of *Helicobacter pylori*-induced gastric diseases. We used an *H. pylori* strain (HP99) in a Korean isolate. Using two-dimensional electrophoresis of protein isolated from *Helicobacter pylori*-induced cells, Coomassie G250 staining and computerized analysis of stained gel, we found proteins whose expression were altered in *Helicobacter pylori*-infected cells as compared to non-infected cells. Intensely stained spots were excised and digested with trypsin, and the resulting peptides were characterized by mass spectrometry (MALDI-TOF). Several differentially expressed proteins were identified by *H. pylori* infection in human gastric epithelial cells. These proteins are related to cell proliferation (prohibitin), molecular chaperones (protein disulfide isomerase, Hsp90), DNA repair (Ku70), and glycolytic enzymes (Triosephosphate isomerase). In conclusion, differentially expressed proteins by *Helicobacter pylori* infection will provide valuable information to understand pathologic mechanism of *H. pylori*-induced gastric diseases including inflammation and carcinogenesis.

Extracellular Matrix (ECM) containing mutated fibrillin-1 (Fib1) down regulates Col1a1 mRNA levels in Tsk1/Tsk1 and +/+ embryonic fibroblasts

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Purpose: It is known that the ECM is able to signal to cells and thereby direct or modulate the transcription of certain mRNAs. This signaling plays an important role in tumor invasion and metastasis, wound healing, remodeling of the ECM and cell differentiation. There are several mechanisms whereby the ECM signals cells to change their metabolism: (1) Receptor molecules binding to specific domains in the ECM; (2) Direct phagocytosis of the ECM molecules or domains into the cell; (3) Structural changes of the ECM domains. We report the effect of an ECM containing either Tsk-mutant or normal Fib1 on the transcription levels of several ECM mRNAs.

Methods: Tsk1/Tsk1 and +/+ mouse embryonic fibroblast cell lines were used. Tsk/Tsk cells produce only mutated Fibrillin-1 which arises from mRNA containing a perfect in-frame duplication of exons 17–40. To test the effect of the ECM containing mutant Fib1, cells of the Tsk1/Tsk1 and the wild type (+/+) genotype were each grown on an ECM produced by either Tsk1/Tsk1 cells or by wild type cells (+/+). The embryonic cells were genotyped by Northern analyses for Fib1 and grown to confluence. The cultures were then harvested and the cells removed, leaving the matrix in the flasks. Matrices produced from Tsk1/Tsk1 and from +/+ cells were reseeded with either Tsk1/Tsk1 cells or +/+ cells. The cells were allowed to grow on the matrices until confluence after which total RNA was harvested. Northern analyses were performed for Fib1, type 1 and V collagens, and GAPDH.

Results: The Northern analyses showed that the steady state levels of mRNA for Col1a1 were modulated by the type of matrix on which the particular cells were grown. When the normal cells were plated on the homozygous matrix, the Col1a1 levels were reduced to 75% compared to when they were plated on the matrix produced by normal cells. When the homozygous cells were plated on the matrix produced by normal cells, the Col1a1 levels were increased by 230% compared to when they were grown on the matrix produced by Tsk/Tsk cells. In contrast the mRNA levels for Fib1, Col5a2 and GAPDH were not changed, indicating that this effect was not global but was specific for Col1a1.

Conclusions: We hypothesize that the mutated Fib1 with its many additional EGF-calcium binding regions and Tgfβ binding domains may (1) affect the homeostasis of the ECM by binding additional growth factors or (2) may present a radically different ECM-3-dimentional architecture. Either or both of these changes could signal the cell to produce less Col1a1.

High-performance peptide MS/MS scoring functions. Applications beyond regular database searches: eukaryotic genome searches, quantitative and semi-quantitative proteomics

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We rapidly introduce a method of designing efficient peptide scoring functions aimed at identifying peptides in protein databases on the basis of their fragmentation spectra. Such scoring functions yield low false positive rates when searching regular protein databases. In this talk we report results obtained when applying these functions to solve less common problems in proteomics: (1) To search eukaryotic genomes and find peptides across intron/exon boundaries. (2) To derive semi-quantitative information when comparing samples. (3) To automate the processing of data in quantitative proteomics comparisons *via* stable isotopic labelling.

The cysteine *S*-conjugate *β*-lyase bioactivation pathway

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Many electrophiles are detoxified through the mercapturate pathway: electrophile → glutathione *S*-conjugate → cysteinylglycine *S*-conjugate → cysteine *S*-conjugate → N-acetylcysteine *S*-conjugate (mercapturate) → excretion. However, if a good leaving group is attached to the sulfur in the cysteine *S*-conjugate, the mercapturate pathway may be diverted by cysteine *S*-conjugate

β-lyases:

$$RSCH_2CH(NH_3^+)(CO_2^-) + H_2O \rightarrow CH_3CH(O)CO_2^- + NH_4^+ + RSH$$

If the eliminated fragment (RSH) is unreactive, the parent cysteine *S*-conjugate is not generally toxic. However, if RSH is reactive, the parent cysteine *S*-conjugate (e.g. *S*-(1,2-dichlorovinyl)-L-cysteine, DCVC; *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine, TFEC) may be toxic. TFEC and DCVC, the cysteine *S*-conjugates of tetrafluoroethylene and

trichloroethylene/dichloracetylene, respectively, give rise to fragments (RSH) that are strong thioacylating agents, particularly of protein lysine residues.

At least ten mammalian pyridoxal 5′-phosphate-containing enzymes catalyze cysteine *S*-conjugate β-lyase reactions. All are normally involved in amino acid metabolism, but catalyze non-physiological cysteine *S*-conjugate β-lyase side reactions. TFEC targets mitochondrial proteins in rat kidney *in vivo* and in cells in culture. The α-ketoglutarate dehydrogenase complex (KGDHC) and aconitase are especially vulnerable to TFEC. Mitochondrial aspartate aminotransferase (mitAspAT) is an important cysteine *S*-conjugate β-lyase. KGDHC, aconitase and mitAspAT are thought to be part of a supra-molecular complex (metabolon) that facilitates substrate channeling. Possibly, mitAspAT within the metabolon facilitates thioacylation of an important lysine group on KGDHC. Cysteine *S*-conjugate β-lyase reactions may contribute to mitochondrial dysfunction associated with aging and neurodegeneration.

Mercaptoethanesulfonic acid hydrolysis of protein in order to determine the tryptophan content

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Deliberation of tryptophan (Trp) from the peptide chain without significant loss is a great challenge for scientists. When the hydrolysis was carried out with 6 M HCl at 105°C for 24 hours, the Trp content of the samples was almost completely decomposed. When other acidic methods were investigated, hydrolysis with 3 M mercaptoethanesulfonic acid gave significantly higher recovery ($P < 0.01$) than that of 3 M p-toluenesulfonic acid containing 0.2% tryptamine in case of the next samples: maize, soybean, milk powder and meat meal. The applicability of the high temperature – short time mercaptoethanesulfonic acid hydrolysis was investigated in order to reduce the time requirements for this step. The recovery of free Trp, pure proteins (lysozyme, cytochrom C, bovine ribonuclease) and that of milk powder with high reducing sugar content were determined related to the control method (125°C, 24 h) with the application of different temperatures (160–180°C) and time periods (15–90 min). At the high temperatures the recovery decreased continuously in the function of the time thus the hydrolysis of the proteins was almost completed when the shortest time was applied that is 45 min at 160°C, 30 min at 170°C, and 15 min at 180°C. At these conditions the decomposition of the Trp related to the control method was not significant. Though the Trp content of the carbohydrate containing samples has been reported to decompose under acidic conditions (6 M HCl), the recovery of the Trp in milk powder was not lower than that of pure proteins at 160°C and at 170°C when 3 M mercaptoethanesulfonic acid was applied as hydrolyzing agent.

Complementary investigations of whey proteins: Instrumental analyses and immunochemical methods

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Whey proteins are important food proteins due to their high nutritional value and their interesting technological properties but also from a health perspective, as they constitute potent allergens. During food processing, e.g. heat treatment or fermentation, they can undergo various physicochemical and molecular changes that might also impact their allergenic potential.

Different instrumental analyses and immunochemical characterisations of whey proteins, with a special focus on β-lactoglobulin, will be presented. Investigations were carried out using liquid chromatography-mass spectrometry (LC-MS) and capillary electrophoresis (CE) as well as enzyme linked immunosorbent assays (ELISA) and western blotting. The complementarity of these methods in assessing the properties of (modified) whey proteins will be discussed.

Besides the analysis of the native proteins in various milk products the effects of two processing conditions were investigated:

(1) The frequently applied heat treatment of milk products is known to cause glycosylation of the whey proteins. The changes occuring during such thermal stress events both on the level of protein chemistry and on the level of allergenicity have been studied in detail. Results obtained by LC-MS and ELISA measurements will be reported.
(2) The fermentation of milk can reduce the resistance of -lactoglobulin against peptic digestion and hence diminish the allergic reaction *in vivo*. The kinetics of peptic digestion of fermented and non-fermented milk products were monitored by CE and pronounced differences were observed.

Peptide antibiotics (Peptaibiotics) of *Trichoderma* species and their potential for bioactivity and biodiversity research

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Peptaibiotics are linear peptide antibiotics composed of 5–20 amino acids. These compounds are exclusively biosynthesized by fungicolous, plant- or entomopathogenic fungi thus assuming a potential importance in the parasitic life cycle of the producers. This hypothesis is supported by membrane-modifying, ionophoric activities of these compounds. The nonribosomal biosynthesis of these compounds results in the presence of different non-proteinogenic marker amino amino acids, such as α-aminoisobutyric acid (Aib) and isovaline (Iva).

Different species of Trichoderma exhibiting strong antimicrobial activities against the causing agents of Eutypa, Esca and Petri disease of grapewine (Vitis vinifera L.) were investigated. After rapid and selective extraction and work-up of six days old petri-dish cultures using a C18 Sep-Pak cartrige, the presence of peptaibiotics was investigated by a combination of LC-coupled ESI-Ion-Trap CID-MS-, MS/MS- and MSn-experiments on putative pseudo-molecular ($[M+H]^+$) and diagnostic fragment ions, selectively searching for the presence of Aib (m/z 85) and Iva (m/z 99) as marker amino acids. Mass spectra are compared with literature data and our own database that was created to enhance screening efficiency. Using this method we analyzed different strains of defined species investigating the presence and (partial) primary structures of peptaibiotics produced with the aim (i) to characterize these compounds which may have a potential importance for the bioactivity of these strains and (ii) to discuss the pattern of peptaibiotics produced as a fingerprint for chemotaxonomic analysis.

Using this approach, we found that two Trichoderma brevicompactum strains (temporary nos. 113 and 114) produce a microheterogeneous mixture of short-, medium- and long-chain peptaibiotics. The structurally novel, short-chain octameric compounds, a mixture of homologues and positional isomers, were shown to possess a N-terminal Ac-Aib-Gly, whereas the C-terminus consists of either free valine or isovaline,

respectively. The long-chain compounds are homologues of alamethicins F30 which are known for their strong antimicrobial activity, including antifungal action.

Three Trichoderma asperellum strains (temporary nos. 193, 194 and 232) produce a diverse mixture of trichotoxin-like compounds which also exhibit strong antifungal properties.

Thus, the compounds detected by LC-ESI-MS/MS could, at least partially, account for the activity of the producing strains against Eutypa, Esca and Petri disease of grapewine. The sequences of the peptaibiotics detected might be used for (re)-classification of Trichoderma species aggregates.

Identification of serum peptides by magnetic bead based fractionation combined with LC-MALDI-TOF/TOF

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The discovery and identification of naturally occurring peptides and proteins reflecting the status quo in biochemical mechanisms and pathways gain more and more importance in context with diagnosis of diseases (e.g. cancer). MALDI-TOF mass spectrometry is a unique tool for the detection of such peptides within complex profile spectra and their subsequent identification. Fractionation, enrichment and purification of peptides and proteins from complex biological material necessary for MALDI-TOF analysis is mandatory and can be achieved by magnetic bead based chromatography. Identification of discovered peptides can be directly accomplished by MALDI-TOF/TOF analysis, whereas larger proteins may not be identified using this approach as protein digestion is required to identify them.

A new strategy combining the magnetic bead based chromatography and LC-MALDI-TOF/TOF analysis allows the further specific enrichment of peptides and proteins and the identification of a higher number of molecules within the low-molecular mass range by MALDI-TOF/TOF analysis. Various magnetic beads with different functionalities (e.g. hydrophobic, cation exchange) were used for peptide and protein capturing from human serum samples. One part of the eluate recovered from the magnetic beads was used for direct acquisition of profile spectra and the other part was analyzed on a capLC system. The eluate was spotted into discrete spots on a MALDI target. All sample fractions were analyzed automatically by MALDI-TOF/TOF mass spectrometry. Database searching allowed the identification of all peptides with molecular weights of approx. less than 3000 Da.

In the presented approach, peptide markers were discovered in MALDI MS profiles obtained from magnetic beads at high sample throughput, while their identification was achieved from a low number of LC-runs using TOF/TOF measurements.

A combined approach to enhance periplasmic expression of human growth hormone in *Escherichia coli*, using a modified signal peptide from *alpha amylase* gene of *Bacillus licheniformis*

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The *alpha amylase* gene signal peptide, originated from a strain of *Bacillus licheniformis*, was shown to be able to transport its native protein, when expressed in *E. coli*. the competence of the fusion protein being processed and translocated through the inner membrane is highly dependent on the amino acid sequences in the signal peptide. Therefore, in order to increase the expression efficiency of BLA signal peptide, we reconstructed the BLA signal peptide coding fragment with the following modifications. Two rare codons of Arg_6 (CGG) and Arg_{10} (CGA) and codons for Leu_{15}(TTA) and Pro_{23}(CCT), in the signal peptide were substituted with their corresponding *E. coli* major codons. Two other changes, including Phe_{20}(TTC) → Leu_{20}(CTG) and Ala_{28}(GCG) → Met_{28}(ATG), were also introduced to increase the processing efficiency. The hGH-expressing plasmid equipped with the modified BLA (BLAF2) was subjected for further expression analysis in a T7-based expression system. We compared the expression pattern of the recombinant bacteria, induced with either IPTG or various concentration of lactose. The results obtained from the protein patterns of the induced bacteria indicates in high expression level of hGH pre-protein (hGH::BLAF2) followed by efficient transfer of the mature hGH to *E. coli* periplasm.

Derivatisation of the arginine residue and its guanidine group with malondialdehyde: Application to peptide and protein analysis by mass spectrometry

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The selective derivatisation of certain amino acids plays an important role in the field of peptide and protein analysis for example for the introduction of isotope labels for relative quantification coupled with selective enrichment of the analytes.

Our work is focused on the development of labelling strategies directed to arginine residues. Therefore we have investigated a tagging procedure based on the reaction of the guanidine group of the arginine side-chain with malondialdehyde under strongly acidic conditions where a stable pyrimidine ring is formed.

The procedure has been applied to a variety of analytes like standard peptides, arginine-rich peptides and tryptic digests of proteins. Quantitative modification can be achieved within approximately one hour but for further analysis the reaction mixture has to be purified.

We investigated the influence of the label on the guanidino group changing its strong basicity and polarity and thus the effect on the retention behaviour of peptides in RP-HPLC. Especially small hydrophilic and arginine-rich peptides often show poor retention resulting in low sensitivity or they are not detected at all. The introduction of the significantly more hydrophobic pyrimidine label seems to alleviate this problem.

For peptide sequencing by LC-MS the high proton affinity of the arginine side-chain sometimes has a negative influence on fragmentation. Thus, we will discuss the influence of the arginine labelling procedure on peptide charge states and fragmentation in LC-ESI-MS/MS. Preliminary results showed that for some peptides more fragment ions were indeed formed after labelling which gives rise to a richer information for de novo sequencing strategies.

Over expression of secB protein in Escherichia coli enhances the periplasmic expression of human growth hormone

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Among several proteins involved in the secretion pathway of proteins in *E. coli*, secB plays a key-important role in solubilization of preproteins before processing. In order to increase processing of a human growth hormone precursor (pelB::hGH) which appeared to have problem in processing efficiency, as a possible solution a regulated co-expression

of a secB gene was considered. In this regard, we designed an arabinose-regulated secB expressing plasmid compatible with an IPTG/lactose-regulated pelB::hGH expressing plasmid.

For the construction of the secB expressing plasmid the origin of replication and antibiotic resistant gene (Amp) of a pBAD vector was replaced by a p15A-Ori and a Kanamycin resistant gene, respectively. The expression and processing of pelB::hGH preprotein in the two-plasmid containing bacteria in a secB over-expression state was compared to that of the pelB::hGH expression in normal bacteria. Although a decline in total expression level of hGH during the over-expression of secB was observable, probably due to presence of two different expressing plasmids, but both the processing efficiency of pelB::hGH and the transport of mature protein into the periplasmic space was enhanced during prolonged arabinose induction. By optimization of the inductions of the secB and pelB::hGH expressions, coordinated with the host growth conditions, enhancement of the expression of mature hGH in the periplasm is expected.

Deletion of 30 amino acids at the N-terminal region of the cystathionine gamma-synthase enzyme causes methionine overproduction

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Cystathionine γ-synthase (CGS), the first enzyme of the methionine biosynthesis in higher plants plays an important role in the biosynthesis pathway, regulating the level and metabolism of methionine in the plant cells. As a response to methionine level, the expression of this enzyme is regulated *via* amino-acid sequences located at its N-terminal part. We demonstrated using reverse-transcription PCR and ribonuclease protection analysis that in addition to the full-length CGS transcript, a deleted form is exists in Arabidopsis. The deleted transcript of CGS lacks 90 or 87-nt located internally in the regulatory N-terminal region of CGS, and it keeping the reading frame of the protein. In order to study the function of this deleted form of CGS we over-expressed it alongside the full-length *Arabidopsis* CGS in transgenic tobacco plants. We found that the transgenic plants expressing the deleted form of CGS accumulated methionine to a much higher level as compared to those expressing the full-length CGS. In order to analyze if like the full length CGS, the transcript level of the deleted form is modulated by methionine level, transgenic tobacco plants expressing the deleted form were irrigated with methionine. Unlike the expression of the full length transcript, which was drastically reduced by this treatment, no response was detected for the deleted form. Therefore, although produced from the full length CGS, the transcript of the deleted-form is insensitive to methionine application.

Integrated proteomics workflows for protein identification and quantitation

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The coupling of liquid chromatography (LC) with mass spectrometry has proven to be a powerful tool for comprehensive proteome analysis. ESI has been used online with LC for the high speed analysis of complex protein mixtures for some time successfully. The new MALDI-TOF/TOF technology, however, coupled to LC became a promising alternative since the temporal constraints for MS/MS precursor ion selection were removed. In the further advanced approach described here, the two MS techniques were used simultaneously, with the goal to increase the sequence coverage of a protein to account for as much of its structure and PTMs as possible.

Digested protein samples were separated by capillary LC and post-column 1:1 split. Peptides were analyzed online by ESI-MS/MS and the other half was spotted onto a 600 μm AnchorChip MALDI-target for offline MS and MS/MS analysis on a MALDI-LIFT-TOF/TOF instrument. MS and MS/MS spectra of selected precursor ions were obtained using a high capacity ESI-ion trap with a scan rate of 5 spectra in 3 sec. The database search results from Mascot were utilized for subsequent MALDI-MS/MS analysis so that the acquisition or redundant information was avoided. The MS and MS/MS data acquired by online-ESI- and offline-MALDI-MS/MS analysis were merged for a joint database search. New software package was developed to control the complex workflow and to handle the results.

A tryptic digest of 3 proteins was used initially to establish the method and the software required to control the process. Even though the scan rates of the ESI ion trap provided fast acquisition of MS/MS spectra, the ability to selecting all precursor ions for MS/MS analysis was restricted by the chromatographic peakwidth and the complexity of the mixture. As ESI and MALDI analyses typically provided complementary information, i.e., some peptides showed up in ESI others in MALDI spectra, complementary sequence information was obtained for each of the 3 proteins. Since the MALDI-MS/MS decision making does not suffer any time constraints, this set-up allowed the intelligent acquisition of only those peptides that have not been successfully identified by LC-ESI before. The fractions deposited on the MALDI-target were available for re-analysis even after several days.

Data dependent MALDI-MS/MS analysis following online-ESI-MS/MS provided a significant increase in sequence coverage. Chromatographic conditions were varied as well as the deposition process.

The goal of these experiments is the ability to approach 100% sequence coverage by MS and MS/MS from isolated proteins, such as the ones isolated from 2D gels. That level of detail is required for unambiguous identification of splice variants, modifications or polymorphisms or other sequence variations in proteins and therefore essential for detailed protein structure elucidation.

The new integrated approach was successfully applied to the identification of yeast proteins from a pull-down assay that were previously not identified with other methods. Therefore, this approach appears to provide additional capabilities to investigate complex protein mixtures at greater depth. MALDI-TOF/TOF measurements were used in this intelligent workflow as to validate and extend the online-ESI results but avoided extensive acquisition time and redundant information.

Integrated LC-ESI-MALDI-MS/MS on a Disposable MALDI target platform

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The sensitive detection of post-translational modifications (PTM) in low abundant peptides from protein digests requires high protein sequence coverage, a high sensitivity of the detection system and a low cross-contamination in between LC-MALDI runs. In an integrated approach a capLC system (Agilent 1100) was coupled using a 1:1 post column split to a fast scanning ESI-ion trap mass spectrometer and to a robot for fraction spotting onto a MALDI target. Following the online LC-MS/MS analysis by the trap and database searching, off line LC-MALDI-TOF/TOF analysis was performed in dependence of the ESI-trap data. MALDI was used 1) to provide additional peptide identifications that were not observed in previous ESI analysis, 2) to validate unsafe ESI matches, under conditions of full automation.

The goal of these experiments is to approach 100% sequence coverage by MS and MS/MS from isolated proteins, such as the ones from 2D

gels. That level of detail is essential for an unambiguous identification of splice variants, PTMs, polymorphisms or other protein sequence variations, i.e. a detailed protein structure elucidation. As PTMs such as sulfation or phosphorylation can be present at sub-stoichiometric levels, the detection of low abundant peaks for the downstream acquisition of MS/MS spectra must safely exclude the possibility of MALDI target cross-contaminations from previous runs. Therefore, disposable AnchorChip MALDI targets were used here. These plastic sample plates must still fulfill the demanding requirements of surface flatness and electrical conductivity for colinear MALDI. They are prespotted with α-cyano-4-hydroxycinnamic acid matrix to allow the direct application of the LC-eluate onto the target. 100 amol detection sensitivity was achieved on these matrix anchors as they concentrate the analyte from the larger droplets that were deposited.

Data dependent MALDI-MS/MS analysis subsequent to online-ESI-MS/MS provided a significant increase in sequence coverage. As an LC-run is immobilized on a MALDI target, the deposited fractions were available for re-analysis even after several days if careful data analysis suggested some to be particularly important to the final result. The disposable targets are particularly useful for extensive sample archival and the influence of archival time vs. data quality was evaluated.

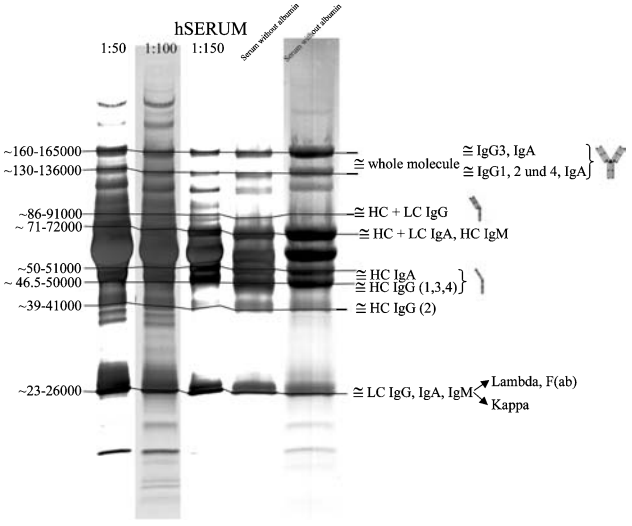

Fig. 1. 1D-PAGE of h-serum immunoglobulins

Topography of immunoglobulins G, A and M on 1D- and 2D SDS page

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Introduction: 2D PAGE of untreated serum/plasma samples frequently implicate orientation problems, mainly due to the albumin content and to the overlapping spot distribution of serum immunoglobulins, both representing the major protein fractions of human serum. So for blotting experiments, e.g. to test the antigeneity of specific antibodies, an orientation aid may save identification time and prevent misinterpretation due to cross reactive precipitations with other than immunoglobulin fragments.

Method: Location an identification of immunoglobulin fragments was done by using different specific antibodies for Western blotting after 1D and 2D electrophoretical separation under reduced conditions. 2D-electrophoresis was performed according to Görg et al. with modifications as described by Bjellquist et al. In brief: first dimension (1D-electrophoresis) was carried out on individually precast IPG-strips with a pH-gradient ranging 3–10 (Immobiline Dry Strip 110 mm, Pharmacia). The first dimension was run under Silicon oil for 70 kVh. The second dimension, a horizontal SDS-Page was done gel containing a resolving zone with a 8–18% total monomer gradient (Excel Gel 8–18, 110 × 245, Pharmacia) using polyacrylamide buffer strips (Tris/Acetat/Tricine discontinous buffersystem).

Visualisation of proteins was done by silver staining. Electroblotting: was performed using a semidry blotting apparatus. The transfer was performed under discontinous and continous [18] conditions at 1.4 mAh/cm^2 at room temperature. After the transfer membranes were washed with TRIS buffered saline and stained with Amido Black 10B or Indian Ink.

Detection of antigens by immunostaining: free binding sites on the membrane were blocked over night with non fat milk. After washing with TRIS buffer saline Tween 20 (TBST) the membrane was incubated with the supernatant at a dilution of 1:100 (in TBST-1% non fat milk) for 24 hours. The membrane was incubated for 10 hours with anti-human IgG conjugated with alkaline phosphatase as the second antibody. As a dye Fast Red TR/Naphtol AS-MX and NBT/BCIP was used.

Results: Figure 1 gives the immunoglobulin bands after 1D separation. 2D electrophoresis of serum samples showed that the light chains

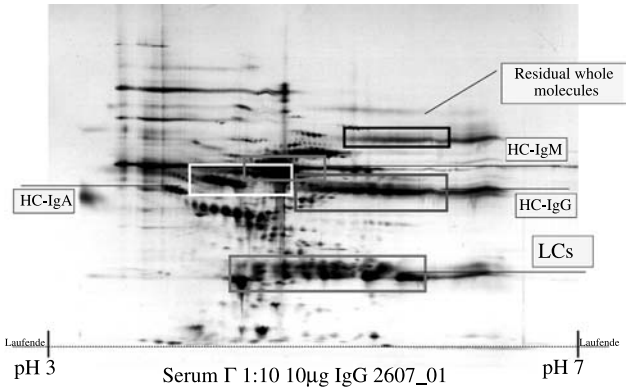

Fig. 2. 2D-PAGE, whole serum

(LC) of all three immunoglobulin isotypes are located within a 4.5–6.5 pI interval with an average MW of 25 kDa. Heavy chains (HC) are located within a MW interval, as expected from 1D electrophoresis, ranging from 55–65 kDa. HC-IgG can be found between 5.0 and 6.5 pI, whereas HC-IgA and HC-IgM could be found at a more acidic pH interval. HC-IgA: 4.0–5.0 and HC-IgM was in between IgA and IgM, e.g. around pI 5.0, but the latter was partly hidden by albumin (for details see Fig. 2).

Resume: Results show that in case of untreated (prepurified, albumin reduced) serum samples all LCs, IgG-HC and IgA-HC easily can be used for Western blotting reactions, whereas IgM-HC is strongly masked by albumin.

Impact of genetic variabiliy on congenital cataract: Elucidation of modified proteins by 2-DE/MS proteomics

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Individual diversity and accordingly individual susceptibility to disease is extensively attributed to the inimitable genetic makeup of

organisms within a population. Gene activity yields proteins, the major class of physiological effector molecules, making proteomics ideal for the investigation of congenital diseases, genetic polymorphism and other genetic problems.

We used ampholyte carrier based 2-DE to separate three independant preparations each of the wild type and alpha 3 connexin KO cataract mutant 10 day old lenticular proteomes of two mouse strains, C57BL and 129SvJ, into around 1000 spots. The KO genotype leads to onset of the cataract phenotype in the 129SvJ background while C57BL KO animals remain healthy. We thus realize the impact of genetic variability on the development of congenital disease in one experiment. Detailed comparative analysis of the 2-DE resolved proteomes allows the visual comprehension of biological networks on the protein species level, a criterium not met by LC/MS. All crystallins ubiquitous to mammals were identified by mass spectrometry and twenty spots appearing differentially between strains and/or mutants were excised and their protein constituents identified by tandem mass spectrometry, resulting in first steps towards understanding the impact of genetic variability on cataract development and possibly inhibition.

Biomarker discovery, identification and quantitation applied to the brain

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This presentation will describe the application of chemical stable isotope labelling strategies coupled with multidimensional separation and tandem mass spectrometry for the discovery of potential biomarkers related to diseases of the brain.

This technology uses a 4-plex set of isobaric tags for peptide derivatization following protein digestion. In mass spectrometry spectra, peptides labelled with any of the tags are indistinguishable. They have identical masses after tagging. Upon fragmentation by tandem mass spectrometry, signature ions are produced that give quantitative protein expression information. Typically changes in protein expression of greater than 20% can be measured confidently. Significantly, ion currents for the sequence informative ions are additive (no splitting), such that the peptide backbone fragments represent the cumulative ion current from up to four samples. Thus, both protein quantitation and identification are simultaneously enabled.

We will demonstrate the use of these chemistry-based approaches in the profiling, identification and quantitation of putative and known biomarkers in brain tumours and in the comparative analysis of diseases causing dementia.

Screening for sequence aberrations and modifications on the proteome level

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2-D PAGE followed by mass spectrometry is an outstandingly useful technique, as the detailed protein structure can be elucidated on the level of each individual spot. Even more information can be obtained from the combination of complementary MS techniques. Then, various features can be illuminated as shown in this approach. This is facilitated by an integrated software environment that comprehensively organises information on all analysis levels.

Total protein was separated on a 2 D gel. After colloidal coomassie staining, the gel was mechanically fixed using a frame equipped with a transponder. The protein spots were excised and transferred to microtitre plates, which were protected against dust and evaporation in dedicated, transponder-equipped plate holders. There, the proteins were digested by porcine trypsin. For high throughput analyses a MALDI-TOF/TOF instrument was used. ESI Ion trap analysis was performed subsequent to nano LC separation (75 um column). Electronic transponders allowed tracking of the complete sample information and status throughout the whole process.

A three step strategy was employed in a fully automatic workflow (''WARP''). (1) Peptide Mass Fingerprints (PMFs) were acquired first and based on the results. (2) MALDI-TOF/TOF was used to verify identifications and increase identification yields using MS/MS analyses. This strategy was also extended to the targeted analysis of peptides that remained unrelated to identified proteins. That approach allowed targeting unexpected structural features such as modification or mutations. (3) For all unidentified or uncertain spots a more powerful yet more time-consuming technique was used: nano LC-MS/MS.

Resulting in high sequence coverage, nano LC-MS/MS was the method of choice for most detailed structural characterisation. Unexplained LC-MS/MS compounds were submitted to automatic *de-novo* sequencing and internal homology searches for the detection of sequence aberrations and posttranslational modifications.

Application of microarrays for functional proteomics

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Microarrays have emerged as powerful in functional Genomics and Proteomics. While DNA microarrays are widely used for simultaneously monitoring the changes in RNA expression levels, the use of peptide and protein arrays is not as wide as the use of DNA microarrays so far.

In our contribution we will present the generation and application of peptide and protein arrays. The use of peptide arrays for an in-depth analysis of a Calmodulin-peptide complex[1] will be discussed. The contribution of different side chains to the overall stability of a Calmodulin-peptide complex is analysed in depth.

There after two different strategies for the generation of protein microarrays are presented. The application of protein microarrays for the identification of kinase targets and binding partners of E100 proteins partners of will be demonstrated.

A peptidyl-aminoacyl-L/D-isomerase from frog skin

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Several bioactive peptides derived from insects, mollusks, amphibians and even primitive mammalians are known to contain a D-amino acid. In general, these peptides are ribosomally synthesized as all-L-precursors. In course of their posttranslational maturation a single L-amino acid at a specific position is converted to its D-isomer.

From skin secretions of *Bombinae*, we have isolated an enzyme which catalyses the isomerization of an L-isoleucine in position two of the antimicrobial peptide Bombinin H to D-allo-isoleucine. The reaction proceeds without the addition of a cofactor. When performed in tritiated

water, radioactivity is incorporated into the second position of the product which argues for a deprotonation/protonation mechanism. It seems likely that one or both of the reactive bases are histidines since the isomerization reaction is inhibited by diethyl pyrocarbonate. The amino acid sequence of this isomerase could be deduced from cloned cDNA and genomic DNA. Apparently, the enzyme is synthesized as part of a huge precursor protein. In expression experiments, injections of cRNA coding the enzyme into *Xenopus* oocytes yielded detectable isomerase activity in the supernatant. Polypeptides related to the frog skin enzyme are present in several vertebrate species including man. This raises the intriguing possibility that peptides containing a D-amino acid are also present in these species.

Lipopeptide ligands and Toll-like receptors

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Cell activation by lipoproteins and shorter lipopeptides is induced through their interaction with Toll-like receptors (TLR) representing a molecular link between host defense mechanisms and microbial products. Ligands for TLR2/TLR6 and TLR2/TLR1 heterodimers are bacterial lipoproteins and lipopeptides. Synthetic analogues of the N-terminal part of these lipoproteins constitute potent immunoadjuvants *in vivo* and *in vitro*. Immunisation experiments with lipopeptide-epitope constructs lead to a long-lasting protection against virus challenge and efficient priming of virus-specific cytotoxic T cells. Potent immune responses were obtained by immunization with conjugates of lipopeptide, hapten and haplotype-specific T helper-cell epitopes.

Fully synthetic combinatorial S-[2,3-bis(palmitoyloxy)-propyl]-cysteinyl peptide collections containing defined amino acids and fully degenerated amino acid positions were synthesized and investigated in cellular assays. Deconvolution led to defined lipopeptides with improved activity. Additionally lipopeptide collections differing in number, length and structure of amide and ester bound fatty acid residues attached to S-[2,3-dihydroxypropyl]-cysteine were synthesized and investigated for biological activity. The dependence of TLR mediated cellular response on the chirality and oxidation state of the S-[2,3-dihydroxypropyl]-cysteine was investigated as well as influences of modifications of the this core structure.

Fluorophore-labeled lipopeptide tools were used to investigate their interaction with living cells.

Antagonists of TLRs such as lipolanthionine peptides were synthesized as potential lead structures for the treatment of symptoms of inflammation and septic shock.

Complexity of microbial proteomes

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We applied complementary methods to model systems starting with a complexity of 16 proteins for proteasomes and ending with *Mycobacterium tuberculosis* with about 4000 predicted genes. The aim is to get access to as much as possible of the protein species present in the proteome under analysis. All of the proteins predicted for the proteasome were identified on 2-DE gels. But already at this low complexity level it becomes clear that proteins are massively modified: There are about 70 spots representing different protein forms from which only some of them were identified at the protein species level. The mycobacterial proteome of a cell extract of late growth phase state was analysed by 2-DE/MS and revealed 1800 spots with 379 different proteins identified (10% of the predicted genes). Isotope coded affinity tag (ICAT)-LC/MS revealed 60,000 peptide masses with 619 (15% of the predicted genes) identified proteins. Because of the overlap of 158 proteins showing the high complementarity of 2-DE/MS and ICAT-LC/MS the proteome is covered at present by 21%. Proteomics also revealed six new genes not predicted by bioinformatics and corrected the N-termini of the gene predictions. For *Helicobacter pylori*, a pathogenic bacterium with about 1550 predicted genes we revealed with a prefractionation strategy about 520 different proteins (33% of the predicted proteome). One of the proteins determined as vaccine candidate was successful as vaccine in animal models and is now in a clinical study. In Berlin a proteome 2-DE database was established (http://www.mpiib-berlin.mpg.de/2D-PAGE), which contains data of more than 10 different bacterial strains. During all of our investigations it became clear that proteomics does not end with the identification of a protein. At present the identification of the protein species, which needs 100% sequence coverage is very time consuming and affords further technology development. Today, the enormous information content of a 2-DE gel is only accessible to a small extent.

Circular dichroism study of bacteriorhodopsin mutants and heterochromophoric analogs

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Bacteriorhodopsin (bR), a major protein of the purple membrane (PM) of Halobacterium halobium, functions as a light-activated proton translocase. bR is one of the best studied membrane proteins. The native chromophore, all-trans retinal, is bound to the ε-amino group of Lys-216 *via* a protonated Schiff base (PSB). In the PM bR molecules are arranged in clusters of three (trimers), which form two-dimensional hexagonal crystals.

Biphasic circular dichroism (CD) spectrum is known as a signature of bR structural organization in the PM. It was initially explained as a superposition of an intrinsic positive Cotton effect arising from the protein environment and a bisignate CD due to exciton coupling (EC) within the bR trimer. However, within last two decades many experimental data testify against EC.

In order to shed light on this controversial subject and to determine the origin of the bisignate CD spectra of native purple membrane, we have explored several approaches. First, CD study of the apo-membrane regenerated stepwise with two chromophores (heterochromophoric bR) was undertaken to verify weather CD of heterochromophoric bR samples meet the requirements/rules of exciton coupling theory.

The second approach was to investigate a conformational flexibility of a single retinylidene chromophore per trimer in the membrane, for which *a priori*, intra-trimeric excitonic interaction is practically excluded. For that we obtained partially (33%) regenerated PM and subjected it to different conformational distortions by using various concentrations of osmotically active agents (sucrose and glycerol). Third, CD study of several bR mutant derivatives was performed.

Our results support the idea of *conformational heterogeneity* of the chromophores inside bR in the trimer, suggesting that three bR subunits in the trimer could be not conformationally equal. Therefore, the bisignate CD spectrum of bR in the purple membrane occurs rather due to a superposition of the CD spectra from variously distorted bR subunits in the trimer than due to inter-chromophoric excitonic interactions.

Recombinant human alpha-1-proteinase inhibitor: Glycosylation, stability and biological activity

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Human plasma-derived α_1-PI is an FDA licensed product, used for augmentation therapy. Currently there are 3 licensed plasma-derived products available for patients with hereditary α_1-PI deficiency for i.v. treatment.

Human alpha-1-proteinase inhibitor (alpha-1-PI) is a well-characterized protease inhibitor with a wide spectrum of anti-protease activity. It belongs to the serpin (serine protease inhibitors) superfamily. Human alpha-1-PI is a 52 kDa glycoprotein comprised of a single polypeptide chain with three carbohydrates attached at asparagines residues. The secondary structure features 9 α-helices, 3 β-sheets (A, B, and C), and a reactive site loop. As a serine protease inhibitor, alpha-1-PI is uniquely designed to perform its functional role. Human α_1-PI is a highly glycosylated, and its glycosylation pattern seems to have a crucial impact on protein stability and therefore, physiological activity.

Over wide spectrum of academic research and R & D work during last years, human gene for α_1-PI has been expressed in various hosts (E. coli, yeasts, plant cells and transgenic animals). However, due to modified glycosylation patterns, a poor stability and, therefore, low biological activity of such proteins, have been a consistent problem impeding progress in the field.

All the above underscores the necessity for a better understanding of structure-function relationships for recombinant glycoproteins as potential therapeutics. As FDA, we intended to evaluate the critical steps of the recombinant α_1-PI production both glycosylated and non-glycosylated and the issues related to low stability of the recombinant α_1-PI. Therefore, α_1-PI may serve as an example to address the problems in the field of recombinant therapeutic glycoproteins. To assess the structure-function effects of glycosylation on stability and biological activity of α_1-PI we comparatively investigate recombinant non-glycosylated and differently glycosylated α_1-PI versions, vs. human plasma-derived α_1-PI and its enzymatically deglycosylated form.

Recombinant human alpha-1-proteinase inhibitor: expression in *Aspergillus niger*

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Human alpha-1-proteinase inhibitor (alpha-1-PI) is one of the major protease inhibitors in human plasma. Its deficiency is associated with development of progressive emphysema. Currently, alpha-1-PI is available for replacement therapy as an FDA licensed plasma-derived product. However, robust viral inactivation steps, which clear known viruses, cannot fully eliminate the risk for emerging viruses. Recombinant alpha-1-PI (r-alpha-1-PI) provides an attractive alternative. Although r-alpha-1-PI has been produced in several hosts, protein stability has been an issue, primarily due to lack of glycosylation or altered glycosylation patterns. We have explored the possibility of expressing the gene for human alpha-1-PI in filamentous fungus *Aspergillus niger*, a system reported to provide "mammalian-like" glycosylation. Our expression strategy features fusion of alpha-1-PI with a strongly expressed, secreted leader protein (glycoamylase), separated by a KEX2-like sequence that provides *in vivo* cleavage. SDS-PAGE, Western blot, ELISA, and activity assay enabled us to select the transformant(s) secreting glycosylated, biologically active r-alpha-1-PI with yields up to 50 mg/L. Currently we are working to achieve enhanced production of r-alpha-1-PI *via* multiple insertions of the expression cassette into the *Aspergillus niger* chromosome. We plan to use this expression system in the future for other recombinant therapeutic glycoproteins.

Crystal structure of truly uncomplexed G-actin

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Many structures of actin solved by X-ray crystallography are available at the present time. Because actin tends to polymerize under crystallization conditions, all of these structures represent actin complexes with other proteins or small molecules that prevent polymerization. Nearly all of these have been shown to stabilize G-actin in a closed conformation, so it is conceivable that the solved structures represent an altered actin conformation rather than that of free actin in solution. Actin can be specifically cleaved at a single site on its DNAse binding loop by ECP32 protease, rendering it non-polymerizable in the Ca-ATP bound form. Such a modification of actin is quite distinct in that it increases the rate of nucleotide exchange and the spectrum of actin's conformational flexibility. We have succeeded in crystallizing and solving the structure of ECP32 cleaved actin to 1.9 Å resolution. Surprisingly, the overall structure is similar to the numerous complexed forms solved previously. Taken together with the other structures, this finding provides the strongest evidence to date that the actin structure observed in crystalline state is that of the preferred conformation of G-actin in solution.

InGaP and InCeP databases: a paradigm for identifying functions of mKIAA/KIAA proteins

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We have previously launched InGaP database (Integrative Gene and Protein expression database; http://www.kazusa.or.jp/ingap2): a comprehensive database of gene/protein expression profiles of mKIAA related to hypothetical ones obtained in our cDNA project. Information about each gene/protein consists of cDNA microarray analysis, subcellular localization of the ectopically expressed gene, and several experimental data using the anti-mKIAA antibody such as Western blotting and immunohistochemical analyses. Further advancement of our project, we are now preparing to release InCeP database (Intracellular pathway database based on mKIAA protein–protein interactions; http://www.kazusa.or.jp/incep/index.html). Novel protein–protein interactions were identified by the combination of our "library" of antibodies and immunoprecipitation followed by mass spectrometric analysis. The core of each pathway was generated from these experimental evidences and further spreading of the pathway was achieved by manual curation of referred data mainly obtained from published articles. KIAA cDNAs and their mouse counterparts, mKIAA cDNAs were mainly isolated from cDNA libraries derived from brain tissues, we thus expect our databases to contribute to the field of neuroscience. In fact, cDNA microarray analysis reveals nearly half of our gene collection is predominantly expressed in brain. Immunohistochemical analysis of the mouse brain provides functional insight into the specific area and/or the specific cell type of the brain. The InGaP database is a seamless database integrating the fundamental genomic and proteomic fields of information and the

InCeP database will yield novel pathways and theories in the field of neuroscience.

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Subcellular fractionation strategies for the proteomic analysis of tissue samples

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In order for proteomics to reach its full analytical potential it is required to establish subcellular fractionation strategies for the individual compartments of a eukaryotic cell. The reason for this is mainly the low expression of certain proteins that play a key role in the metabolism. Two compartments are of special interest in the light of biomarker identification by proteomic methods: the cell nucleus and the cellular membranes. In this respect, the proteomic analysis of subcellular fractions originating from frozen tissues is of great interest. However, the enrichment of such fractions from frozen human or animal material requires special protocols.

We have established two nuclear extractions and one total membrane isolation protocol for the isolation of the desired subcellular fractions from frozen mouse livers. The quality of the applied protocols was assessed by a gel-free proteomic approach (MudPIT) and by Western blots with cellular marker proteins. Both nuclear fractions are shown to be highly enriched in their corresponding proteins (46% and 54% nuclear proteins vs. 6% nuclear proteins in the total cell lysate). The fraction designated as the membrane fraction contained 61% membrane proteins vs. 9% in the total cell lysate.

The subcellular fractionation strategies presented here are crucial steps to further enhance the application range of our ProteoSHOP toolkit of gel-based and gel-free proteomics technologies.

Protein expression profiling of MYC- and TP53 related pathway in human oral squamous cell carcinoma by using proteome analysis

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Two-dimensional protein gel electrophoresis technology was used to systematically characterize the protein expression profiles of oral squamous cell carcinoma (OSCC) at cellular process levels in order to investigate the multiple interactions of different expressed proteins, which are potential involved in tumor growth and metastasis. The proteome from the center of ten squamous cell carcinoma tissues compare to the surrounding mucosa as control one, was profiled from each patient. In the frame of this study, we were able to identified 23 different expressed proteins (16 up regulated and 7 down regulated) of more than 356 identified different gene products, which was classified into different functional sets according to there gene ontology. Of these functional profiling, down regulations of the proteome were only shown in cell adhesion, cell motility, organogenesis, enzyme regulator and structural molecule activity that are supposed to play important roles in pathophysiology of the tumor. In contrast, the up regulation was mainly shown in the purine and pyrimidine metabolism as well as in the p38 MAPK, PI*K/AKT and Death receptor signaling pathway. Eight from 16 up regulated gene products, was found to be linked to 3 main locus genes TP53, MYC, and MYCN and the corresponding pathway is discussed in detail. Taken together, potentially significant pathogenetic cellular processes were identified and indicated, that the up regulated functional profiling has a significant impact on the tumorgenesis *via* TP53, MYC and MYCN pathway. We are also able to show, that the gene ontology-combined with the proteome-analysis can help us to study the complexity of expression profiles of two-dimensional protein analysis *via* its cellular process-level approach.

Conformationally constrained amino acids as a tool for designing selective analogues of bradykinin

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In the present work, sterically constrained non-coded amino acid, 1-aminocyclohexane-1-carboxylic acid (Acc), was substituted in position 8 of the peptide chain of bradykinin (BK) and positions 6, 7 or 8 of its B_2 receptor antagonist [D-Arg^0,Hyp^3,Thi^{5,8},D-Phe^7]BK, previously synthesized by Stewart's group, in order to reduce the flexibility of the peptides, thus forcing the peptide backbone and side chains to adopt specific orientations. Knowing that acylation of the N-terminus of several known B_2 blockers with a variety of bulky groups has consistently improved their antagonistic potency in the rat blood pressure assay, the Acc substituted analogues were also synthesized in N-acylated form with 1-adamantaneacetic acid (Aaa). The activity of eight new analogues was assayed in isolated rat uterus and in rat blood pressure tests. The results clearly demonstrated the importance of the position in the peptide chain into which the sterically restricted Acc residue was inserted. Meanwhile Acc at positions 6 and 7 led to reduction of antagonistic qualities oreven restored the agonism, respectively. Acc at position 8 enhanced antagonistic qualities in both tests. The Acc at position 8 of BK strongly reduced the agonistic potency. In most cases acylation of the N-terminus led either to enhancement of antagonistic potencies or to further decrease of agonistic potency. Our findings offer new possibilities for designing new potent and selective B_2 blockers

From phage-display to GPCR antagonists: discovery and characterization of highly potent FPRL1/CCR12 peptide antagonists useful for target validation

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The human Formyl-Peptide Receptor Like 1 (FPRL1) belongs to the Gi Protein-Coupled Receptor (GPCR) family. Recently, some studies suggest the FPRL1 receptor may interact with structurally diverse ligands associated with different inflammatory diseases, i.e. Prion disease, Alzheimer's disease, Amyloidosis. up to day, no potent FPRL1 antagonist was available to characterize the physiological and pathological role of this receptor. From a Phage-Display approach, we found two 12-mers peptides (PXL001 & PXL004) as antagonists of this receptor. Those peptides were further

characterized and confirmed as potent FPRL1 inhibitors in various assays, e.g. cAMP, FLIPR, GTP-γ-S and on different cell-lines, i.e. recombinant, neutrophils. In neutrophil migration assays, PXL001 and PXL004 block the migration induced by 100 nM of WKYMVM (known as a FPRL1 agonist) with IC50 closed to 100 nM and 50 nM, respectively. In preliminary results, we also found that our peptides are potent inhibitors of sCKβ8-1, a recently characterized natural agonist of FPRL1. This original approach helps us to discover potent peptide antagonists, to identify pharmacophoric elements within those peptides that could serve as starting points for molecular modeling and the development of small molecule inhibitors. The discovered/characterized peptides will be used to validate FPRL1/CCR12 target in Alzheimer disease.

Amino acid analysis of various collagen-types targeting hydroxylated prolyl and lysyl resisdues

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Whereas the gene sequences code only twenty amino acids, several hundred co- and posttranslational modifications, mostly as a result of sequence-specific enzymatic activity, can regulate proteins. In mammals, for example, the majority of all proteins are modified, which affects their physical and chemical properties, folding, conformation, stability, activity and function. This study targets hydroxylation of proline and lysine in collagen. Collagen forms a heterogeneous group of various sequentially and structurally related proteins, which are called collagen types. Typical for all collagen-types is their triple-helical structure formed by three collagen chains containing a high degree of glycine and proline residue necessary to stabilize the helical structure, which is further stabilized by hydroxylation of specific residues within the sequence. The distribution of hydroxyproline (Hyp) and hydroxylysine (Hyl) within the sequence as well as the stereochemistry of the Hyp-residues are still not well understood.

The goal of this study was to establish a sensitive amino acid analysis to identify the Hyp- and Hyl-content in various collagen types using HPLC-techniques coupled on-line to an ESI mass spectrometer. The difficulty of the separation system results from the four Hyp-isomers found in collagens, that is cis-3- and trans-3-Hyp as well as trans-4- and cis-4-Hyp. Moreover, all four Hyp are isobaric to each other and to Leu and Ile complicating their identification by mass spectrometry. Thus it is obligatory to separate at least these six amino acids by liquid chromatography. We describe a pre-column derivatization with an analogue of marfey's reagent, i.e., N^2-(5-fluoro-2,4-dinitrophenyl)-L-valine amide (L-FDVA), followed by separation of all proteinogenic amino acids as well as the Hyl- and Hyp-isomers by reversed-phase (RP-) HPLC and detected by UV and ESI-MS. Furthermore, the cis-3-, trans-3-, trans-4-, cis-4-Hyp and Hyl ratios in different collagen types were determined.

NMR studies on 45 proteomics targets of Helicobacter pyroli

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The H. pyroli proteins of which molecular weight are around 10 kDa were selected for the investigation of their 3-dimensional structures. 45 target genes of H. pyroli were selected and cloned into E. coli expression vector. pET/BL21 (DE3) was chosen as expression system of target proteins. 32 genes of 45 target genes were successfully expressed in the expression system. 20 expressed proteins could be obtained in soluble fraction after cell lysis. Initially, we tried to characterize the 20 soluble target proteins using CD (circular dichroism) and gel chromatography, of which data were used for optimization of NMR solution conditions. Among the 20 target proteins, 16 proteins have been characterized with CD spectroscopy until now. All these characterized 16 proteins were studied with NMR spectroscopy. 1H-15N HSQC spectra of 16 samples were obtained. The spectra of 4 samples could not be obtained since they aggregated and precipitated during concentration. 10 samples showed good HSQC spectra and 4 samples showed promising spectra. However 2 samples showed bad spectra. NMR spectra of 6 targets were acquired with double (15N, 13C) labeled proteins and backbone assignments could be obtained. We could determine 3 dimensional structures of 4 target proteins. These structures will be discussed.

Arginine as target for tagging and trapping strategies for mass spectrometry-based protein and peptide analysis

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Arginine residues in proteins are not only important for various biological functions but also for different analytical approaches in proteomics.

Trypsin, the most frequently used proteolytic enzyme, cleaves proteins C-terminal to Arg (and Lys) residues so that approximately half of all resulting tryptic peptides will carry this strongly basic residue. This has implications for mass spectrometric analysis since the guanidino group of Arg is a preferred protonation site and also influences the fragmentation behavior of the peptides. Chemical modification of arginine residues is an interesting strategy to potentially improve the quality of tandem MS spectra, and a novel approach followed by our group will be presented.

In addition, we have previously developed an arginine-specific tagging procedure that involves the reversible modification of the guanidino group with 2,3-butanedione and an arylboronic acid. This concept has now been expanded to capture arginine-containing peptides from mixtures like tryptic digests. This is achieved by binding the Arg-peptides to affinity materials containing immobilized phenylboronic acid moieties in the presence of butanedione. After the removal of arginine-free sample constitutents, the captured peptides can be released by a pH switch to acidic conditions where the boronic ester is unstable. We see this method as an alternative to current strategies that mostly rely on the enrichment of cysteine-peptides, especially for mixtures of moderate complexity.

The same tagging reaction has now also been applied on the intact protein level, where the reactivity of individual arginine residues can be correlated with their solvent accessibilities as calculated from 3D structures.

Improved detection of phosphopeptides using new CID fragmentation techniques

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In proteome research the analysis of protein phosphorylation is of particular interest as this type of highly dynamic post translational modification is a widely used process for regulation of protein function. However the detection and characterization of Serin and Threonin phosphorylation in CID experiments is often difficult, as CID results in neutral loss of phosphate with little if any further fragmentation of the peptide chain itself. In addition many phosphorylated peptides have the tendency to further loose water during a second fragmentation step. To gain further sequence information new CID approaches were used.

As a model system with well defined concentrations, digests of standard proteins were spiked with different substoichiometric amounts of synthetic phopho peptides. These peptides were derived from tryptic peptides of natural occurring phosphorylated proteins) and analyzed using nano LCMSMS using ion trap MS with different neutral loss triggered fragmentation options (HCTultra, Bruker).

Different approaches of neutral loss triggered fragmentation techniques were compared: MS(3) of the resulting mass, MS(2) with additional fragmentation on the neutral loss masses but without prior isolation, to save time and gain sensitivity. To overcome sequential loss of water/phosphate an MS(2) approach using consecutive fragmentation of all expected neutral losses within one single MSMS spectrum was used. The latter bears the risk of fragmenting fragment ions instead of neutral loss peaks, thus deteriorating spectra quality, but it turned out to be method of choice for certain types of samples. The limit of detection for all three approaches has been compared using standard samples as well as digests of natural phosphorylated proteins.

The role of AP (atmospheric pressure) – MALDI low energy CID (collision induced dissociation) and vacuum MALDI low as well as high energy CID in proteomics

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Mass spectrometry (MS) in the tandem MS (MS^2) and multistage MS (MS^n $n \geq 2$) turned out to be an indispensable tool in proteomic research and protein characterization. The combination of ESI or nano ESI in the *on-line* or *off-line* mode with various types of analyzers seems to have reached a mature state, whereas vacuum MALDI, intermediate vacuum MALDI and atmospheric pressure MALDI (AP-MALDI) coupled to tandem and multistage MS analyzer is at its infancy. Particular the possibility of high energy collision induced dissociation (CID) seems to open up new opportunities, which has been limited in the past to not anymore available 4-sector tandem instruments. Of importance is that MALDI, in which form ever, can be connected in a semi-automatic fashion to separation techniques without any time constrains.

In-gel digested proteins, generated during proteome studies, were analyzed by AP-MALDI and vacuum MALDI for peptide mass fingerprinting (PMF) and the results were evaluated in terms of sequence coverage as well as sensitivity. PMF is in many cases not sufficient for protein identification and therefore MS sequencing by means of CID experiments was performed. Low energy (LE) CID experiments were performed by means of a hybrid multistage vacuum MALDI-ion trap (IT)/two-stage reflectron (RTOF) or an AP-MALDI-3D-IT instrument. In parallel high energy (HE) CID was done on a prototype vacuum MALDI TOF/curved field RTOF mass spectrometer. All these sequence data (LE as well as HE CID data derived from singly-charged precursor ions) were compared and evaluated in terms of length of sequence tags, sensitivity, suppression effects and finally complete information content (e.g. differentiation Leu/Ile, tandem stage vs. multistage (MS^3) experiments) for subsequent bioinformatics input.

Protein PEGylation: an attempt to control the extent of reaction

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The poly(ethylene glycol) (PEG) attachment to therapeutic proteins has recently become an expanding field of interest for protein drug delivery. PEGylation offers an extended *in vivo* half-life that is likely due to the increased size and steric hindrance invoked by the PEG. By changing the solution environment, these properties of the PEG could potentially be manipulated towards favoring and optimizing a target degree of PEGylation. The myoglobin PEGylation reaction with a linear 30 kDa PEG was challenged by different solution environments such as various pH, salt, and/or cosolvents. Although the kinetics could be altered, the changes were not preferential for the monoPEGylation reaction. The maximum amount of the target degree of monoPEGylated protein remained comparatively constant, suggesting minimal alterations of the conjugate stereochemistry as a function of solution conditions. To best explain our data a simple PEGylation reaction model was proposed that includes a non-specific PEG/protein complex and a conformational equilibrium between the *worm* and *shell* structures of monoPEGylated species. In conclusion the flexibility of using multiple solution conditions offers desirable latitude in developing optimal PEGylation conditions.

Protein microarrays – Technology and applications

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Following the completion of the human genome sequencing project the combination of DNA microarrays with sophisticated bioinformatics allows scientists to take a global view into biological systems. Now, in today's proteome era, the time is ready for protein microarrays for screening entire genomes for proteins that interact with particular factors, catalyse particular reactions, act as substrates for protein-modifying enzymes and for target identification in the pharmaceutical industry.

Protein microarrays are miniaturised and parallelised assay systems that allow a simultaneous determination of several parameters with only minimal sample consumption. On planar protein microarray systems hundreds to thousands of different capture molecules can be immobilised in rows and columns onto the surface. Beside planar microarrays, bead-based flow cytometry approaches are a very interesting alternative, especially when the number of parameters of interest is comparably low. Bead-based assay systems employ colour-coded microspheres as a solid support for the capture molecules. A flow cytometer identifies individual bead types and quantifies the amount of captured target on each individual bead. Sensitivity, reliability and accuracy are similar to those observed with standard microtiter ELISA procedures.

However, the basic requirement for protein microarrays is the availability of appropriate capture and detection molecules, e.g. antibodies. High numbers of binders can be enriched by classical ways (immunisation and hybridoma cell lines) and recombinant library technologies (phage display, ribosomal display and mRNA display). Currently, not the selection process, but the characterisation of the obtained binding molecules represents the bottleneck in the generation process of these molecules.

We have developed protein microarray-based methods to determine the properties of isolated binders. These methods allow to screen a large number of binders for selectivity, specificity and affinity. In this presentation an overview will be given on the current stage of protein microarray technology with a special focus on miniaturised multiplexed immunoassays for antibody characterisation.

Strain-dependent regulation of plasticity-related proteins in the mouse hippocampus

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Strain distribution in behavioral and cognitive tests is well-documented in literature.

An effect of strain on the expression of proteins critically involved in synaptic plasticity, learning and memory has not been described. We

have been addressing this question by determining expressional levels of a panel of proteins involved in neuronal information processing in hippocampus of five mouse strains by immunoblotting. Four inbred strains, commonly used for generating genetically modified mice and for conventional experiments in pharmacology and toxicology and one outbred mouse strain have been subjected to a standard protocol for mouse phenotype assessment. Basal and protein levels following behavioral testing have been determined.

A significant effect of strain was detected for total and phosphorylated calcium-calmodulin dependent kinase IIα (CaMKII, pCaMKII), phosphorylated mitogen-activated protein kinase (pMAPK), total and phosphorylated calcium-responsive element binding 1 (creb, pcreb), early-growth response protein 1 (egr 1), brain derived neurotrophic factor (BDNF), drebrin and postsynaptic density-95 (PSD-95). Moreover, a significant effect of behavioral testing was observed for CaMKII, pcreb and PSD-95. Statistical analysis revealed significant interaction between the effect of strain and behavioral testing for pCaMKII, pcreb, egr 1 and PSD-95. In summary data presented herein highlight the importance of careful selection of the mouse strain when synaptic plasticity-related principles are the targets of analysis, specifically with respect to hippocampal protein expression. Moreover, results showing the effect of behavioral testing on protein expression imply a new aspect to be considered for the experimental design and interpretation of studies investigating synaptic plasticity at the behavioral and biochemical level.

Tools for sample preparation and fractionation in 2D electrophoresis

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Two-dimensional gel electrophoresis (2DE), with its unique capacity to resolve thousands of proteins in a single run, is a fundamental research tool for nearly all protein related scientific projects. Over the last two decades, numerous researchers in academia and in industry have improved the technology to the point where a novice user is capable of achieving respectable gel separations on the first try. In addition, 2DE technology as practiced today has seen enormous gains in reproducibility, resolution and automation all of which contribute to its widespread use. Nevertheless, 2DE is still a technically demanding method.

The quest to map and characterize each and every protein in a given cell type, tissue or organism has given 2DE an additional boost as the separation methodology of choice for many proteomics laboratories. However, the task list for a proteomics researcher is daunting: the number of proteins in a biological sample, although unknown at this time, is believed to be in the 100,000s, covering a concentration range of 7 or more orders of magnitude. In addition, the proteome is extremely dynamic, with protein expression depending on the cell state and further complicated by posttranslational modifications such as phosphorylation or glycolysation, to name just two possible changes to proteins in a functional biological system.

As more and more laboratories start up their own proteomic effort or ramp up existing programs, they realize that meticulous attention to 2DE methodology is only one critical aspect when identifying differentially expressed proteins or investigating a particular biological pathway.

The information content of 2DE is heavily influenced by a proper sample preparation strategy. Interestingly, not much attention was paid to this area during 2DE methodology development.

This talk is meant to provide a broad overview of the principles and recent developments of sample preparation tools prior to the first step of 2DE. Examples from three strategies for sample preparation, based on solution chemistry, chromatography and electrophoresis, will be discussed in detail as well as used to illustrate how these key areas can be applied to general-purpose sample cleanup and sample fractionation for enrichment of low abundance proteins.

Application of capillary zone electrophoresis (CZE) with polyamine-modified capillaries for analyzing proteinaceous allergen preparations

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In Europe, 15% of the population is affected by pollen-related allergies. Currently available allergen products lack a well-defined composition. Since immunogeneity is measured on basis of reactivity with IgE antibodies, different mixtures might result in comparable potencies. Thus, the European Commission has launched a multi-disciplinary program, called CREATE, to promote standardization of allergen products by providing certified reference materials (CRMs) as a medium-range target. Therefore, the characterization of preliminary candidate reference materials and the monitoring of different preparation approaches is mandatory and requires the development and validation of quantitative methods with excellent separation efficiency. Capillary zone electrophoresis (CZE) meets these requirements. Beside the separation electrolyte and the pH, the separation temperature and voltage have been optimized systematically. Substantial improvement in allergen separation was achieved by adding 1-2 $mmol \cdot L^{-1}$ of a polyamine agent directly to the electrolyte. By attachment of the polyamine additive to the surface of the separation capillary, adsorption of allergens onto the capillary wall was reduced. In addition, the electroosmotic flow (EOF) is efficiently suppressed. The elaborated method was finally applied to characterize available candidate reference materials, namely the major birch pollen allergen Bet v 1, the olive-allergen Ole e 1 and the Timothy allergens Phl p1 and Phl p5. Compositions of allergen preparations differed substantially depending on their manufacturing protocol. Up to 13 different fractions were identified in some standards. Sample consumption per analysis was only 18 nL, corresponding to less than 200 fmol of total protein. Results indicate a repeatability for the effective mobility of allergens expressed as CV $<0.25\%$ ($n = 6$). Presented results will show that CZE greatly surpasses other analysis methods, such as size-exclusion chromatography, in terms of separation efficiency and quantitation and is highly suitable for determination of purity and qualitative make-up of purified allergen preparations.

* Presentation of poster.

Functional comparison of dystrophin and utrophin

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Dystrophin and utrophin are homologous proteins serving to link the actin cytoskeleton to the extracellular matrix. This link is believed to be important in membrane stabilization and, in the absence of dystrophin, sarcolemmal integrity is compromised, leading to muscular dystrophy. Overexpression of utrophin has been shown to functionally replace dystrophin in the *mdx* mouse model of Duchenne muscular dystrophy. Genetic strategies to replace defective dystrophin with utrophin require full characterization and direct comparison of these proteins. We have directly compared the actin-binding properties of full-length utrophin and dystrophin expressed in baculovirus expression system. Our results suggested that dystrophin and utrophin both bind laterally alongside actin filaments through contribution by the spectrin-like repeats, but the rod domain epitopes involved differ between the two proteins. In working to directly compare molecular epitopes involved in dystrophin and utrophin interaction with actin, we have expressed several recombinant fragments encoding the amino-terminal domain of dystrophin or utrophin and different numbers of spectrin-like repeats. We further characterized the actin binding properties of each recombinant fragment. Our

results indicate that the amino-terminal domain is essential for utrophin binding to actin, but that the following 10 spectrin-like repeats dramatically enhance the affinity and stoichiometry of the interaction. In contrast, dystrophin binds alongside actin filaments *via* independent and distinct binding sites located on its amino-terminus and middle rod domain. While our results provide a molecular basis for the effective therapies, they are also of interest when searching for common themes within the family of actin-binding proteins.

Investigation of proteins in post-mortem human brain tissue by laser microdissection/pressure-catapult and nano-LC/MSMS

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The molecular mechanisms involved in most neurodegenerative disorders, such as Parkinson's and Alzheimer's disease, are still unclear. So far, protein expression analysis is often performed on homogenized preparations of whole tissues which do not provide any information about relevant changes in specific cell types.

The aim of the following study was to examine whether laser-microdissected samples of single cell types from post-mortem brain tissue can be used for protein expression analysis.

Therefore, we used haematoxylin and eosin stained frozen sections (15 μm) from human postmortem brain tissue and collected cell material selectively by laser-microdissection and pressure catapulting (LMPC) as combined in the *AutoLPC*-function of a MicroBeam instrument (P.A.L.M. Microlaser Technologies). Different amounts of material have been collected such, that the cells are readily fragmented and homogenized while being catapulted out of the brain section into the urea denaturation buffer (6 M urea, 100 mM Tris/HCl), which was placed in the center of a cap for sealed reaction tubes exactly on top of the desired areas for LMPC-cell-harvest.

The tryptic digested protein mixture was subsequently analyzed with a nano-LC/MSMS ion trap system (Agilent Technologies). Database searching was done with SpectrumMill MS Proteomics Software.

The samples were injected to a reversed-phase enrichment column, which was backflushed after 5 minutes loading time. The peptides were then eluted to a ZORBAX SB300 C18 nano column (0.075×150 mm) which was directly coupled to an ion trap mass spectrometer *via* an orthogonal nanospray source. The gradient was raised with 0.25% B per minute resulting in a total cycle time of 200 minutes. The ion trap was operated in Auto-MS2 with ActiveExclusion and SmartFrag for generating MS and subsequent MS/MS spectra of the tryptic peptides.

Applying this non-contact investigation method we were able to indentify the glial fibrillary acidic protein by 25 distinct peptides, the myelin basic protein by 6 distinct peptides and a mutant beta-actin by 7 distinct peptides.

A reducing agent activates human porphobilinogen synthase *via* intrasubunit transfer of a zinc ion

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Human porphobilinogen synthase [EC.4.2.1.24] is a homo-octamer enzyme. In the active site of each subunit, Cys^{122}, Cys^{124} and Cys^{132} are placed near two catalytic sites, and coordinate a zinc ion, referred to as "a proximal zinc ion". Cys^{223} is placed at the orifice of the catalytic cavity and coordinates a zinc ion, referred to as "a distal zinc ion", with His^{131}. To elucidate the role of the distal zinc ions in activation, four mutant enzymes, C122A ($Cys^{122} \rightarrow$ Ala), C124A ($Cys^{124} \rightarrow$ Ala), C132A ($Cys^{132} \rightarrow$ Ala) and C223A ($Cys^{223} \rightarrow$ Ala) were constructed, and kinetic studies, DTNB titration and zinc ion analysis with gel-filtration connected-ICP-MS were performed under oxidizing, air-saturated (0°C, atm) and reducing conditions. A disulfide bond was formed among Cys^{122}, Cys^{124} and Cys^{132} under oxidizing conditions. Zinc ion-free 2-mercaptoethanol increased the activity of the wild type enzyme without a change in the total number of zinc ions, but C223A was not activated. These findings suggest that a distal zinc ion moved to the proximal binding site when a disulfide bond among Cys^{122}, Cys^{124} and Cys^{132} was reduced by 2-mercaptoethanol. Thus, in the catalytic functioning of the enzyme, the distal zinc ion does not directly contribute but serves rather as a reserve as the next proximal one that catalyzes the enzyme reaction. Redox change of the three catalytic cysteines accommodates the catch and release of the reserve distal zinc ion placed at the orifice of the active center.

Cytoplasmatic interaction partners of the neuropeptide receptor SorLA

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The type I transmembrane receptor SorLA/LR11 consists of a large luminal and a short cytoplasmatic part. SorLA belongs to the family of low-density-lipoprotein (LDL) receptors which was recently identified to play a role in signal transduction in addition to its function in ligand internalization. One example is the activation of the ApoE receptor 2 by Reelin resulting in the phosphorylation of the adaptor protein Dab1. In contrast to other members of the LDL-receptor family SorLA contains a Vps10 domain which specifically binds the neuropeptide head activator (HA), a growth factor in the development of the nervous system, and the glial cell line-derived neurotrophic factor (GDNF). A Vps10 domain is also present in Sortilin, a signaling receptor for proNGF which mediates neuronal cell death.

To investigate ligand-induced SorLA signalling we had a look at interaction partners of SorLA's small cytoplasmatic tail. In a yeast two hybrid assay two promising binding partners have been identified and were further characterized. Additionally, we tested whether HA or GDNF binding to SorLA induces the phosphorylation of adaptor proteins or the receptor itself. Therefore, samples of ligand-induced and non-induced neuronal cells were analysed on one hand by 2D-gel electrophoresis followed by a new phosphoprotein staining method, and on the other hand by specific antibodies against activated members of known signalling cascades or against phosphorylated amino acid residues.

Helicobacter pylori induces cyclooxygenase-2 expression and integrin-mediated adhesion in gastric epithelial cells

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Helicobacter pylori (*H. pylori*) infection is an important risk factor for chronic gastritis, peptic ulcer and gastric carcinoma. We previously demonstrated that *H. pylori* induced expression of cyclooxygenase-2 and adhesion molecule integrin α_5 in gastric epithelial AGS cells. Cyclooxygenase-2 (COX-2) and integrins are responsible for tumor cell proliferation, tumor invasion and metastasis in gastric epithelial cells. Proteinase-activated receptor-2 (PAR-2) belongs to subgroup of G-protein coupled receptor family. Present study aims to investigate whether *H. pylori* infection induces expression of COX-2 and integrin *via* PAR-2 activation in gastric epithelial cells. The results show that expression of COX-2 and integrin $\alpha_5\beta_1$ were increased by *H. pylori* infection. AS ODN for PAR-2 suppressed the increase in expression of COX-2 and integrin $\alpha_5\beta 1$ induced by *H. pylori* infection. Overexpressed PAR-2

increased expression of integrin $\alpha_5\beta1$ without *H. pylori*. Trypsin and PAR-2 are constitutively expressed in human gastric epithelial AGS cells. Conclusively, the results suggest that *H. pylori* infection may induce COX-2 and integrin $\alpha_5\beta_1$ expression mediated with PAR-2 activation in gastric epithelial cells, which may contribute to *H. pylori*-induced gastric carcinogenesis.

Nitric oxide as a signaling molecule for the expression of chemokine and chaperone proteins in human gastric epithelial cells

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The regulation of Inducible NO synthase (iNOS) is primarily at the level of transcription. Transcription of iNOS is induced by a variety of stimuli, including lipopolysaccharide, cytokines, and bacteria. Large amount of NO produced by iNOS are harmful to the tissues by producing peroxynitrite, which is a reaction product between NO and superoxide. iNOS activity was enhanced in gastric mucosa of patients with *H. pylori*-positive duodenal ulcers. Our previous study showed that *H. pylori* – induced apoptosis is related to large amount of NO produced by iNOS in gastric epithelial AGS cells. In other hands, expression of IL-8, a chemokine, is highly stimulated by *H. pylori* in gastric epithelial cells. Present study aims 1) to investigate whether NO directly stimulates IL-8 expression, and 2) to determine the differentially expressed proteins by NO in gastric epithelial AGS cells. Northern blot analysis, ELISA, EMSA (electrophoretic mobility shift assay), and luciferase assay were used for mRNA and protein expression of IL-8 as well as the activation of nuclear transcription factors (NF-κB, AP-1, C/EBP). As a result, NO donors, SIN-1 and NOC-18, induced IL-8 mRNA expression dose- and time-dependently. Both NO donors activated NF-κB and AP-1, but not C/EBP, and thus stimulated IL-8 production in AGS cells. Proteomic analysis was applied for determination of the differentially expressed proteins using 2D-separation, Coomassie G250 staining, tryptic digestion and MALDI-TOF analysis. Several cellular stress-related proteins were differentially expressed. The results may contribute the pathophysiologic mechanism of *H. pylori*-induced gastric diseases and other related gastric diseases.

Comparative proteomic investigations of biological protein samples using the quantitative Protein Sequence Tag (qPST) technology

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The Protein Sequence Tag (PST) technology is a gel-free proteomic approach for the analysis of complex protein mixtures. It is applicable to a wide range of samples with different biochemical properties and involves a combination of highly reproducible chemical and enzymatic cleavage steps coupled with the tagging of amino groups using a proprietary labelling reagent. Subsequent peptide analysis and characterisation is achieved by in-depth LC-MS and LC-MS/MS investigations. PST has been further developed to allow for the differential quantitative analysis of complex proteomes through the development of the isotope labelled quantitative PST tags (qPST).

The performance of qPST was evaluated with *S. cerevisiae*, grown on either galactose or ethanol as carbon source. The data obtained from this application will be presented and demonstrate the robustness of the qPST approach in detecting quantitative changes in complex proteomes with

(1) three replicate analyses showing a high reproducibility
(2) low false positive rate
(3) good correlation from the literature.

Subsequently, qPST was applied to the analysis of human plasma samples. The results demonstrating the robustness and utility of the qPST approach will be presented.

In summary, qPST is a powerful approach to discover biologically relevant biomarkers using differential quantitative protein analysis in complex proteomes of cells and body fluids. It provides accurate quantitative determination of differential expressed proteins and can be used to address comprehensive quantitative proteome analysis.

Enrichment of glycoproteins and glycopeptides supported by magnetic particles and detected by MALDI-TOF-MS

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Glycosylation is with at least 50 to 80 percent the most common form of post-translational modification of proteins. Since it turns out that the degree and the type of glycosylation depends on the status quo of cells and is mostly linked to certain diseases the discovery and identification of those modified peptides and proteins gain more and more importance in diagnosis. In particular aberrant or missing glycosylation can be utilized as profiling parameter. MALDI-TOF mass spectrometry is an ideal technique for identifying a large number of peptides and proteins and their corresponding modifications. Enrichment, isolation and purification of glycosylated peptides and proteins from different sources – a prerequisite for MALDI-TOF mass spectrometry – can be attained by affinity chromatography supported by magnetic particles.

Concanavalin A (ConA) and di-boronic acid functionalized magnetic particles were developed to enrich glycosylated peptides and proteins. ConA specifically binds mannosyl and glucosyl residues of polysaccharides and glycoproteins [1] containing unmodified hydroxyl residues at positions C3, C4 and C6 [2] and can be applied as a general tool for capturing of N-glycosylated peptides and proteins. In contrast, boronic acid forms a covalent bond with 1,2-cis-diol group containing molecules [3] like mannose, galactose or glucose. Thereby it additionally facilitates the enrichment of the more heterogeneous O-linked oligosaccharides but not the capture of N-linked oligosaccharides of the complex type.

The functionalized beads were employed to establish and optimize protocols for the binding and detection of glycosylated peptides and proteins with respect to an automated workflow and the subsequent detection and identification by MALDI-TOF mass spectrometry. For several model proteins the capture could be demonstrated by SDS-PAGE and MALDI-TOF mass spectrometry. According to the type of glycosylation, high mannose, hybrid or complex type, the different proteins were enriched by ConA or boronic acid functionalized beads, respectively. RNase B could be isolated from spiked human serum samples by ConA beads according to the well-known N-linked high mannose oligosaccharide structure.

iTRAQ labeling in conjunction with 2-D LC and tandem MS to study the proteome and the dynamics of excitatory synapses in the brain

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Glutamatergic synapses form the major excitatory neurotransmission pathway in the brain, and drive the activity of vast number of neurons. Many of these synapses undergo neuronal activity dependent changes in synaptic efficacy, which are considered as the molecular events that underlie memory and learning. These changes are known to involve

posttranslational modifications of the synaptic proteins, and the alteration of protein composition.

The molecular description of the synapse and its activity dependent dynamics are demanding because this organelle contains high number of membrane proteins and high degree of protein structural and functional interactions. In the first instance we have defined the proteome of the synapse, using a combination of 2-D gel electrophoresis and LC-MSMS approaches. We then explore the usefulness of iTRAQ reagents for global quantitation of the synaptic proteins. Synaptic membranes from two mice strains were separately trypsin digested, and then tagged with iTRAQ reagents. The samples were pooled together, and the peptides fractionated by a cation exchange column, followed by a capillary C18 column. In the second liquid chromatography step fractions were deposited off-line onto an Applied Biosystems MALDI metal target every 15 sec, i.e. 125 nl per fraction. Up to a maximum of 20 peptides per fraction were submitted to MALDI TOF/TOF MS analysis. Our data reveals that iTRAQ labeling of proteins is simple, yields quantitative results, and increases the sensitivity of the MSMS measurement substantially. We are now applying this technique to study the dynamics of synaptic proteins driven by the alteration of neuronal activity.

Proteomics analysis of striatal proteins in a rat model of L-DOPA-induced dyskinesia

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To this day, dopamine-replacement therapy with levodopa (L-DOPA) has been the main treatment for Parkinson's disease. Unfortunately, prolonged treatment with this drug is associated with the development of several side effects, which include abnormal involuntary movements (dyskinesia). To investigate the molecular mechanisms underlying L-DOPA-induced dyskinesia, unilaterally 6-hydroxydopamine-lesion rats were treated chronically with saline, L-DOPA or bromocriptine. Rats were tested on a rodent abnormal involuntary movement scale, which revealed the occurrence of severe dyskinesia in about 50% of the L-DOPA-treated animals. Bromocriptine induce robust motor activation but no dyskinesia. Striatal proteins were isolated and profiled using two dimensional difference gel electrophoresis (2D-DIGE) and mass spectrometry (MS). An average of 2000 spots were compared for statistical differences among all the groups, and 62 spots showed significant changes (ANOVA $p < 0.05$) in protein expression between the different conditions. Identification of the differentially expressed proteins is now in progress using Matrix-Assisted Laser Desorption/Ionization-Time Of Flight (MALDI-TOF) MS and tandem mass spectrometry (MS/MS).

Peptide motif of O,O-diacylated lipopeptides recognized by Toll-like receptor 2/6 heterodimers

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Lipoproteins from mycoplasma and their synthetic S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-(R)-cysteinyl-peptide analogues are strong immune modulators that activate early host responses after infection. These N-terminally free but O,O-diacylated lipopeptides induce signalling in cells of the immune system through Toll-like receptor TLR2/TLR6 heterodimers. To investigate the influence of the peptide sequence on biological activity a combinatorial lipohexapeptide collection was prepared by fully automated solid phase peptide synthesis and Fmoc/tBu chemistry and analysed by HPLC-ESI-MS.

By screening this collection in an *in vitro* IL-8 induction assay with the human monocytoid cell line THP, we systematically evaluated the potential of 19 proteinogenic amino acids in all sequence positions of the peptide moiety to induce cytokine release by interaction with TLR. Amino acids favourable and unfavourable for biological activity were listed in an activity pattern and used as a base for the definition of individual lipopeptides. Unexpectedly most Pam_2Cys-lipopeptides induce IL-8 but those lipopeptides with a proline residue next to the lipoamino acid loose their biological activity.

Protein changes in *Ixodes ricinus* during blood meal degradation

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The Danish tick *I. ricinus* parasitize three different hosts both mammals and birds during the three year life cycle. The aim of this study was to identify the last blood host being the host which the nymph had parasitized before molting to the adult instar. The reason for the study was to reveal the origin of the host contributing the most to the life cycle of the tick and thereby the maintenance of tick-borne diseases in Denmark. The most common tick-borne diseases are Lyme Borreliosis and Tick-borne encephalitis (TBE) causing illness in both animals and humans.

The adult ticks of the experiment were collected from known hosts. First ticks were analyzed by two dimensional gel-electrophoresis (2DE) and MS to examine if some proteins, originating from the host, persisted during the off host period (30 weeks) The results showed that few proteins can reveal, which animal the tick has parasitized. In the results from the 2DE it was found that different muscle proteins were detectable even when the tick had died of starvation. This lead us to change from 2DE to ELISA locating different heat stable proteins e.g. troponin I. With ELISA is detection of many different host species now possible, thereby making it more realistic to trace the primary hosts of the tick.

A plasma membrane proteomic study of a rat model for schizophrenia

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Schizophrenia is a mental illness affecting about 1% of the world population. Symptoms of schizophrenia include psychosis, apathy, social withdrawal, and cognitive impairment such as deficits in working memory. Alterations in the development of temporal lobe structures such as the hippocampus are implicated in this disorder. Accordingly, a rat model with neonatal excitotoxic lesions in the ventral hippocampus (nVH lesion) has been extensively used to study schizophrenia. Post-pubertal anatomical and behavioral phenotypes of these animals show similarities with those of schizophrenic patients. Moreover, alterations in expression of several

plasma membrane proteins have been observed in the prefrontal cortex of adult nVH lesion rats as well as in brains of schizophrenic patients.

Proteomic approaches have proven to be particularly useful for screening of molecular changes in healthy and diseased tissues, including brain tissues. In addition, organelle fractionation preceding proteomic analysis decreases the complexity of protein extracts and may thus increase the possibility to identify less abundant proteins. In this study, plasma membrane enriched prefrontal cortical protein fractions were obtained from post-pubertal nVH lesion rats and sham littermates and proteomic patterns were compared. The identity of proteins present in significantly different spots was revealed by means of MALDI and nano-HPLC-ESI-Q-TOF mass spectrometry, and protein annotations were assessed. The present observations may help elucidate molecular mechanisms relevant or associated to schizophrenia-linked behaviors observed in post-pubertal nVH lesion rats. These findings may contribute to the development of a reference database to investigate schizophrenia-linked protein expression.

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Identification of different expressed tumor-associated proteins in oral squamous cell carcinoma by using proteome analysis

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Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignancy and a major cause of cancer morbidity and mortality worldwide. Globally about 500,000 new oral and pharyngeal cancers are diagnosed annually, and three quarters of these are from the developing world. One main reason for the high prevalence is, that HNSCC is normally a late-diagnosed disease and is usually only discovered when the first complications occur. Therapy of advanced HNSCC in the late state is far away from therapeutic success. Five years survival rates for mouth, tongue, oropharynx and laryngopharyngeal cancers seldom exceed 40%. Therefore, the existence of a reliable, accurate, cost-effective and non-invasive test for HNSCC is desirable.

Proteomics is a promising technology in the identification of proteins. Proteomics approaches have been successfully employed in studies of lung, breast, prostate and gastrointestinal cancer, and in studies of tongue squamous cell carcinoma and adenoid cystic carcinoma cell lines of human salivary gland. Here we applied proteomics technologies to analyze subfractions from HNSCC and control samples, enriched in cytosolic, microsomal and mitochondrial proteins in order to identified different expressed proteins, that may be used as predict diagnostic markers for the early detection of cancer.

A two-dimensional electrophoresis database of rat pheochromocytoma PC12 cells proteome

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PC12 cells are well-documented and widely applied as many kinds of models in neurobiology and neurochemistry studies. Yet a thorough proteomic analysis has not been performed so far. Here we reported the construction of a large scale two dimensional protein database for PC12. The proteins extracted from PC12 cells were separated by two dimensional electrophoresis and identified by the state-of-art matrix-assisted laser desorption/ionization time of flight-time of flight mass spectrometry. 1080 protein spots, excised from three different two-dimensional gels, were identified with high confidence, representing 475 different gene products, mainly binding proteins and enzymes. 297 identified proteins are located in the low molecular weight region below 20 kDa. This database represents today one of the largest two dimensional databases for higher eukaryotic cell proteomes and for low molecular weight proteins. In addition, we confirmed that calcylin in PC12 cells was N-acetylated. The database of PC12 proteome is expected to be a powerful tool for neuroscientists.

Protein profiling and identification of cerulein-stimulated pancreatic acinar AR42J cells by proteomics and genomics

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Cholecystokine (CCK) analogue cerulein causes pathophysiological, morphological and biochemical similarities to various aspects of human pancreatitis. Doses of CCK or cerulein beyond those that cause the maximum pancreatic secretion of amylase and lipase results in pancreatitis, which is characterized by a dysregulation of the digestive enzyme production and cytoplasmic vacuolization and the death of acinar cells, edema formation, and an infiltration of inflammatory cells into the pancreas. Using DNA microarrays together with proteomic techniques (two-dimensional electrophoresis and MS), we evaluated the correlation of mRNA and protein levels in pancreatic AR42J cell lines treated with cerulein. Several spots were identified in pancreatic AR42J cells by two-dimensional electrophoresis and MALDI/TOF MS. Protein disulfide isomerase related protein, dnaK-type molecular chaperone hsp72-ps1, stress-induced phosphoprotein 1, glutamate dehydrogenase mitochodrial precursor, RuvB-like protein 1, heterogeneous nuclear ribonucleoprotein H1, aldehyde reductase 1, Tpi1 protein, ubiquitin thiolesterase, and peroxiredoxin 2 were up-regulated, whereas valosin-containing protein, 78 KD glucose-regulated protein precursor and adenosyl-homocysteinase were down-regulated. A genomic approach using a microarray kit showed that lithostatin, guanylate cyclase 2C, myosin light chain kinase 2, cathepsin C and progestin were induced by cerulein. These proteins and genes are related to cellular stress such as reactive oxygen species (ROS), calcium homeostasis, cell signaling or apoptosis. In conclusion, the differentially expressed proteins and genes will provide valuable information to understand pathophysiologic mechanism of acute pancreatitis and may be used as diagnostic index for human acute pancreatitis.

Functional studies on several nervous system related proteins to understand mechanism of neuroprotection

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Brain injury and neuronal damage in stroke, AD, PD and other related degenerative diseases of nervous system are very commonly occurred in modern society. To understand the mechanism from basic researches on how these diseases happened is critically important for drug development and clinical treatment. To address this question, we will take a look on the viewpoint from functional studies on nervous system related gene including neuroglobin (NGB), Na^+, K^+-ATPase 1 $\beta 2$ subunit (NKA1b2), Netrin-4, *etc*. Neuroglobin is a newly discovered nervous system specific O_2-binding protein and promotes the survival of neuronal

cells upon hypoxia and ischemia. To better understand the molecular mechanism of neuroprotective effects, we used yeast two-hybrid technique to screen for proteins interacting with NGB from a human fetal brain cDNA library using NGB as bait. A candidate clone coding the C-terminal portion of NKA1b2 was identified. The interaction of NGB and NKA1b2 was further confirmed by GST-pull down and by co-immunoprecipitation. Consistent with binding to NKA1b2, NGB inhibits the enzymatic activity of Na^+, K^+-ATPase in a dose and time dependent manner. As partial inhibition of Na^+, K^+-ATPase activity is a potential survival strategy for neuronal cells in early stage of hypoxia, our data revealed a potential mechanism on neuroprotective function of NGB. According to which, it could be seemed that the balance between energy producing and energy consumption based on neuronal-specific expression of neuroglobin and NKA1b2, extensively expressed cytoglobin and eye-specific globin are critically important to neuronal survival. Recently, we are working on the alternative splicing analysis of hundreds of nervous system related genes, i.e. Hypoxia-inducible factor family and Netrin family, to figure out how these genes are involved in maintaining normal functions of nervous system, and most importantly, how the unexpected alternative splicing events occurred in these genes will cause degenerative diseases of nervous system. Therefore, our research will provide novel ideas on the mechanism of neurodegenerative diseases and will possibly open a new window for drug discovery for degenerative diseases of nervous system.

D-Amino Acids, D-Amino Acid Oxidases/Racemases and Racemization

D-Amino acid and racemase in aquatic animals

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Several phyla of marine invertebrates contain a copious amount of free D-alanine in various tissues (3–100 μmol/g wet wt.). These invertebrates include crustaceans, bivalve mollusks (subclass Heterodonta), several polychaete worms, and sea urchins. We have clarified hitherto that in these animal tissues D-alanine, together with L-alanine, is a major osmolyte responsible for the intracellular isosmotic regulation. Free D-alanine and alanine racemase were found in the body wall of several polychaetes but not in the other species. Under salinity stress of *Perinereis aibuhitensis* from 50 to 150% seawater, only D- and L-alanine increased largely, suggesting that only D- and L-alanine works as the effective osmolytes in this species.

Several bivalves belonging to order Arcoida (subclass Pterimorphia) contain free D-aspartate in place of D-alanine. Free D-aspartate and aspartate racemase were detected in the foot muscle of two species of ark shell and also in the nervous tissues of squid and octopus species.

We have already cloned a cDNA of D-amino acid oxidase (DAO) from the hepatopancreas of common carp *Cyprinus carpio*. To clarify the mechanisms of inducible nature of carp DAO, it was overexpressed in *E. coli* and purified to homogeneity. The purified preparation showed the highest activity toward D-alanine with a high k_{cat} of 190 s^{-1} compared to 10 s^{-1} of the pig kidney DAO. From a three-dimensional model and enzymatical characteristics, carp DAO is close to the pig kidney DAO structurally, but analogous to the yeast DAO enzymatically in turnover rate and stabilities.

Pyridoxal-5′-phosphate-dependent aspartate racemase of the bivalve *Scapharca broughtonii* is homologous to mammalian serine racemase and possesses an α,β-elimination activity

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Aspartate racemase (AspR) that we purified from *Scapharca broughtonii* supports the production of high concentrations of D-aspartate present in the bivalve tissues. This enzyme is the first AspR purified from animal tissues and unique in its pyridoxal-5′-phosphate (PLP)-dependence in contrast to microbial AspRs thus far characterized well, which are all PLP-independent. In addition, the enzyme activity is markedly increased and decreased in the presence of AMP and ATP, respectively. To further analyze the structure-function relationship of the enzyme, we cloned the cDNA of AspR, purified and characterized the recombinant enzyme expressed in *Escherichia coli*. The cDNA included an open reading frame of 1.017 bp encoding a protein of 338 amino acids, and the deduced amino acid sequence contained a PLP-binding motif. The sequence exhibited no significant identity to other AspRs and showed the highest identity (44–44%) to mammalian serine racemase (SR) followed mainly by L-threonine dehydratase and its putative homologs. These relationships were fully supported by phylogenetic analysis of the enzymes. Active recombinant AspR found in the *E. coli* extract represented about 10% of total bacterial protein and was purified to display essentially identical physicochemical and catalytic properties with those of native enzyme. Moreover, experiments with the enzyme thus prepared revealed that the bivalve AspR shows an α,β-elimination activity toward L-*threo*-3-hydroxyaspartate and L-serine besides the racemization activity. The observed bifunctionality, in addition to the homology in amino acid sequence, emphasizes a close relationship between the AspR and mammalian SR that also dehydrates D- and L-serine.

D-Aspartic acid is involved in the vision of *Sepia officinalis*

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In this study we report finding high concentrations of D-Asp in the retina of the cephalopods: *Sepia officinalis*, *Loligo vulgaris* and *Octopus vulgaris.* D-Asp increases in concentration in retina and optic lobes as the animal develops. In neonatal *Sepia officinalis* the concentration of D-Asp in the retina is 1.8 ± 0.2 μmol/g tissues and in the optic lobes 5.5 ± 0.4 μmol/g tissues. In adult animals D-Asp is found at a concentration of 3.5 ± 0.4 μmol/g retina and 16.2 ± 1.5 in optic lobes (1.9 times increased in the retina and 2.9 times increased in the optic lobes). In the retina and optic lobes of *Sepia officinalis* the concentration of D-Asp, L-Asp and L-Glu is significantly influenced by the light-dark environment. In adult animals left in the dark these three amino acids significantly drop in concentration in both retina (about 25% reduced) and optic lobes (about 20% reduced) compared to the control animals (animals left in a diurnal-nocturnal physiological cycle). The reduction in concentration is in all cases statistically significant at a P value 0.05–0.01. Experiments conducted in *Sepia officinalis* by using D-[2,3-^{3}H]aspartic acid have permitted us to know that D-Asp is synthesized in the optic lobes and then is actively transported into the retina. D-aspartate racemase, an enzyme which transforms L-Asp into D-Asp is

also present in these tissues, and it is significantly decreased in concentration in animals left for 5 days in the dark compared to the control animals. Our hypothesis is that the dicarboxylic amino acids: D-Asp, D-Asp and D-Glu play important roles in vision.

Amino acid racemization in the course of the Maillard reaction

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We have recently shown that heating of L-amino acids together with glucose or fructose under weakly acidic conditions leads to a fast partial racemization (epimerisation) and formation of D-amino acids. The interaction of reducing sugars and amino compounds is known as the Maillard reaction. It was postulated that intermediates of the Maillard reaction, the Amadori and Heyns compounds, are the precursors of D-amino amino acids. In order to corroborate this hypothesis we synthesized the Amadori compound fructose-L-phenylalanine (Fru-L-Phe). On dry heating at 200°C for 5 min (30 min) 15.5% (30.0%) D-Phe could be detected. Fructose-amino acids with different amino acid residues have been detected in many heated and stored foods, *e.g.* in dried fruit and vegetable, milk products, cocoa beans or soy sauce. Decay of these compounds with release of D-amino acids is dependent on temperature, storage time, water activity, pH, and presence of catalysts. The proposed mechanism represents a new route for the formation of D-amino acids in foodstuffs in general.

Effect of D-amino acids on some mitochondrial functions in rat liver

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We studied the role of the D-amino acids (D-aa) D-serine, D-alanine, D-methionine, D-aspartate, D-tyrosine and D-arginine on rat liver mitochondria. The stability of D-amino acids, mitochondrial swelling, transmembrane potential and oxygen consumption were studied under oxidative stress conditions, hypertension, alcoholism and ammonemia in rat liver mitochondria. In the presence of glutamate-malate all D-aas salts increased mitochondrial swelling, while in the presence of succinate plus rotenone only D-ala, D-arg and D-ser, induced mitochondrial swelling. The transmembrane potential ($\Delta\Psi$) was decreased in the presence of 1 μM Ca^{2+}. In spontaneously hypertensive rats (SHR), D-ser and D-ala augmented mitochondrial lipoperoxidation, compared with the normotensive genetic Wistar-Kyoto rats (WKY). D-ser increased in chronic alcoholism. During ammonemia, only D-ser increased 41% lipoperoxidation. The D-aa studied exerted effects on mitochondria *via* an increase of free radicals production.

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Racemization of tryptophan of food protein influenced by different technologies

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In order to determine the amount of triptophan (Trp) in food samples hydrolysis of protein is necessary. Under the most often used acidic hydrolysis conditions the amount of this amino acid partially decompose due to oxidative processes and the loss is even higher in real food samples when carbohydrates are present. Hydrolysis in alkaline solutions (e.g. barium hydroxide) has been reported to preserve almost the whole Trp content of the sample and this hydrolysis method can be used if the ratio of the enantiomers is not in the scope of interest. In the case of the determination of D- and L-Trp, racemization of Trp excludes this sort of solution for hydrolysis. Among acidic conditions the highest recoveries were reported in case of using 3 M mercaptoethanesulfonic acid and 3 M p-toluenesulfonic acid containing 0.2% tryptamine. The first purpose of the research was to accomplish the hydrolysis of proteins without significant racemization of Trp. The next step was the separation of the enantiomers in order to determine the amount of D- and L-Trp in food samples. First diastereoisomers were formed during precolumn derivatization with a chiral reagent 1-thio-β-D-glucose tetraacetate (TATG) and o-phthaldialdehyde (OPA), and the separation was accomplished on an achiral stationary phase column following fluorescence detection with high performance liquid chromatography (HPLC). The method was used to investigate the rate of racemization of Trp in free and protein-bonded form.

Racemization and isomerization of aspartate in protein naturally occur in various elderly human organs

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It is generally believed that only L-amino acids have a physiological role in species other than bacteria. Recently, the existence of some D-amino acids, particularly D-aspartate (D-Asp), in various organs such as tooth, bone, aorta, brain, eye lenses, skin, ligament and lung of elderly human has been reported. The presence of D-Asp in aged tissues of living organisms is thought to result from the racemization of Asp residues in the polypeptide where the proteins in such tissues are metabolically inert. We previously reported the presence of D-isomers at two specific sites of Asp residues in alpha A- and alpha B-crystallin from aged human lenses. The reaction was also accompanied by isomerization to the beta-Asp (isoaspartate) residues. These amino acid residues occur *via* a succinimide intermediate. Recently, 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 human donors. Furthermore, we found the D-beta-Asp in protein of gastric mucosa from mouse by immunohistochemical analysis. These results suggest that aging and oxidative stress is closely related to the formation of D-beta-Asp in the proteins. The D-Asp formation occurs much more easily in proteins than was thought. We propose that D-beta-Asp could be a useful indicator not only for aging but also for stress.

Astroglial D-amino acid oxidase is the key enzyme to metabolize extracellular D-serine, a neuromodulator of N-methyl-D-aspartate receptor

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D-Amino acid oxidase (DAO) is a flavoenzyme that catalyzes D-amino acids and present in the mammalian kidney, liver and brain. In brain, gene expression of DAO is detected in astrocytes. Among

the possible substrates of DAO *in vivo*, D-serine is proposed to be a neuromodulator of the *N*-methyl-D-aspartate (NMDA) receptor. Several reports indicate that D-serine may play a role in pathological conditions related to dysfunction of the NMDA receptor. Massive stimulation of NMDA receptors was implicated in neural damage following stroke. Hypofunction of the NMDA receptor has also been implicated in the pathology of schizophrenia. It is notable that the novel human gene G72 has recently been implicated in schizophrenia and shown to bind with DAO and up-regulate its catalytic activity. DAO was itself associated with schizophrenia, and the combination of G72/DAO genotypes had a synergistic effect on disease risk. In this context, it is interesting to note that the inhibitory effect of chlorpromazine, one of the classical antipsychotic drugs, on the activity of DAO through competition with FAD, was reported back in 1956.

In search of the physiological role of DAO in the brain, we investigated the metabolism of extracellular D-serine in glial cells. We found that after D-serine treatment, rat primary type-1 astrocytes exhibited increased cell death. In order to enhance the enzyme activity of DAO in the cells, we established stable rat C6 glial cells overexpressing mouse DAO, designated as C6/DAO. Treatment with a high dose of D-serine led to the production of hydrogen peroxide (H_2O_2) followed by apoptosis in C6/DAO cells. Among amino acids tested, D-serine specifically exhibited a significant cell death-inducing effect. DAO inhibitors, sodium benzoate and chlorpromazine, partially prevented the death of C6/DAO cells treated with D-serine, indicating the involvement of DAO activity in D-serine metabolism. In addition, 3-amino-1,2,4-triazole, an inhibitor of catalase, enhanced the cytotoxic effect of D-serine. Taken together, we consider that extracellular D-serine can gain access to intracellular DAO, being metabolized to produce H_2O_2. These results suggest that astroglial DAO plays an important role in metabolizing a neuromodulator, D-serine.

Biochemical dynamics and function of D-aspartate in mammals

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Mammalian D-aspartate (D-Asp) is predominantly present in neuroendocrine and endocrine tissues. In rats, D% (proportion of D-Asp in the total Asp) is approx. 40% in the adrenal gland (3 weeks of age), 30–40% in the pineal gland and the testis (adults), 10% in the embryonic cerebrum and 5% in the pituitary gland (adults). Quantitative analysis shows that the D-Asp expression pattern displays a distinct and unique developmental change in each tissue. In addition, immunohistochemical staining with a specific anti-D-Asp antibody that we have generated reveals that D-Asp is found in specific cells at distinct periods during the development of these tissues.

D-Asp apparently has biological activities in these tissues. In the pineal gland, D-Asp is localized in the pinealocytes and suppresses melatonin secretion. In the testis, D-Asp is predominantly present in mature germ cells and is presumed to act on Leydig cells in a paracrine fashion to increase testosterone production. In Leydig cells, D-Asp stimulates the gene expression of StAR, an essential factor for steroid synthesis. In the pituitary, D-Asp is present in prolactin-producing cells and presumably stimulates prolactin secretion in an autocrine or paracrine fashion.

Our observations also indicate that D-Asp is indeed synthesized in cultured mammalian cells, although the precise synthetic pathway is not presently clear. Endogenous D-Asp is predominantly present in the cytoplasm of cells and is spontaneously and continuously released into the extracellular space through an undefined mechanism. In addition to this mechanism, D-Asp appears to be released through a channel following appropriate agonistic stimulation.

Suppression of protein isoaspartyl (D-aspartyl) *O*-methyltransferase in cultured mammalian cells

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Spontaneous deamidation of L-asparagine residues and isomerization of L-aspartate residues (L-Asp) results in generation of iso-aspartate or D-aspartate residues (iso-Asp or D-Asp) in proteins under physiological conditions of pH and temperature. Protein L-isoaspartyl (D-aspartyl) *O*-methyltransferase (PIMT) can repair the isomerized proteins by initiating the conversion of these iso-Asp and D-Asp to L-Asp. Recent reports have indicated that PIMT-deficient mice experience fatal epileptic seizures that bring about early death at an average of six weeks of age. In addition, the deficient mice accumulate higher levels of iso-Asp (and D-Asp) in their tissue proteins as compared to control mice. These results suggest a pathogenic significance of iso-Asp (D-Asp) and physiologically important role of PIMT for repairing iso-Asp (D-Asp). However, the detailed molecular mechanism by which accumulation of iso-Asp (D-Asp) leads to pathological abnormalities such as neurodegenerative disorders remains unknown. In this study, we isolated PIMT-silenced cell clones from HEK293 cells following transfection of a PIMT siRNA expression vector. We then characterized resultant molecular abnormalities in intracellular signaling after EGF stimulation.

The expression of PIMT in the isolated cells was significantly decreased at both the mRNA and protein levels compared to control cells. Analysis of the PIMT-silenced cells showed that MEM and ERK, members of the EGF signaling cascade, continued to be phosphorylated even 30 min after EGF stimulation, whereas in control cells, EGF induced transient phosphorylation of these proteins with the highest levels reached at 5 min after stimulation. These results suggest that repairing iso-Asp (D-Asp) is required to maintain normal MEK-ERK signaling after EGF stimulation.

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Mammalian D-aspartyl endopeptidase: a scavenger for noxious racemized proteins in aging

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The accumulation of D-isomers of aspartic acid (D-Asp) in proteins during aging has been implicated in the pathogenesis of Alzheimer's disease, cataracts and arteriosclerosis. Here, we identified a specific lactacystin-sensitive endopeptidase that cleaves the D-Asp-containing protein and named it D-aspartyl endopeptidase (DAEP). DAEP has a multi-complex structure (MW: 600 kDa) and is localized in the inner mitochondrial membrane of mouse and rabbit, but DAEP activity was not detected in *E. coli*, *S. cerevisiae*, and *C. elegans*. A specific inhibitor for DAEP was newly synthesized and inhibited DAEP activity (IC_{50}, 3 μM), a factor of ten greater than lactacystin on DAEP. On the other hand, the inhibitor did not inhibit either the 20S or 26S proteasome.

Is there a correlation between age and D-aspartic acid in knee cartilage?

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L-Aspartic acid (Asp) is one of the fastest racemizing amino acids such that the abnormal D-form (D-Asp) has been found in stable biological human tissues such as dentin in teeth, eye lens and brain. Earlier reports showed that there was a linear correlation between age and D-Asp in teeth. We have previously reported at other ACS meetings that we found significant levels of D-Asp in normal and osteoarthritic knee cartilage. Since cartilage is a slow regenerating tissue, we hypothesized that D-Asp should accumulate in knee cartilage and that there might be a correlation between the age of the person and the amount of D-Asp found in cartilage. Our analysis of approximately 100 samples of normal knee cartilage showed that there was only a slight correlation between the age of the person and the amount of D-Asp (nmoles/g). Surprisingly, there was also a slight correlation between age and the amount of D-Asp in the male subjects, but not in the female subjects.

Involvement of D-amino-acid oxidase in the D-serine-induced nephrotoxicity

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D-Serine is nephrotoxic in rats. Intraperitoneally injected D-serine causes necrosis of the cells in the proximal tubules, resulting in the glucosuria, aminoaciduria, proteinuria, and polyuria. D-Amino-acid oxidase is suspected to be involved in this nephrotoxicity because (1) D-serine is reabsorbed in the straight part of the proximal tubules, where cell damages are observed, (2) D-amino-acid oxidase is present in this part, (3) D-serine is a good substrate of this enzyme, and (4) one of the reaction product of D-amino-acid oxidase is cytotoxic hydrogen peroxide. However, there are perplexing problems: (1) D-serine is not nephrotoxic in mice, guinea pigs, rabbits, dogs, hamsters or gerbils which have D-amino-acid oxidase activity comparable to rats, and (2) D-alanine which is a good substrate of D-amino-acid oxidase is not nephrotoxic in rats. These problems cast doubt on the involvement of this enzyme in D-serine-induced nephrotoxicity.

Since we have found a mutant rat strain lacking D-amino-acid oxidase, we have examined whether D-amino-acid oxidase is involved in the D-serine-induced nephrotoxicity. When D-serine was intraperitoneally injected into control rats, glucosuria and polyuria were provoked. However, D-serine was injected into the mutant rats that did not have D-amino-acid oxidase, glucosuria or polyuria was not observed at all. When D-propargylglycine, which is known to exert its nephrotoxicity through the metabolism of D-amino-acid oxidase, was injected into the control and the mutant rats, glucosuria and polyuria were observed in the control rats but not in the mutant rats. Therefore, these results indicate that D-amino-acid oxidase is responsible for the D-serine-induced nephrotoxicity.

D-aspartate in rat salivary glands

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Free D-aspartic acid (D-Asp) has been reported to occur in wide variety of tissues and cells, exclusively in central nervous system and endocrine tissues. In this study, we demonstrate that large amounts of D-Asp are present in the rat exocrine tissue, salivary glands. In adult male rats, D-Asp concentrations in parotid and submandibular gland were 212 ± 68 and 233 ± 34 nmol/g wet weight, respectively, and was low (38 ± 20 nmol/g wet weight) in sublingual gland. This result indicates that substantial level of D-Asp exists not only in central nervous system and endocrine tissues but also in exocrine tissues. D-Asp concentration in parotid gland increased transiently at 3 weeks of age and decreased thereafter. In contrast, the D-Asp level in submandibular gland continued to increase gradually from 1 to 7 weeks of age and remained at an adult level after 7 weeks of age. D-Asp concentration was almost constant at low level during 1 to 18 weeks in sublingual gland. Using anti-D-Asp antibody, immunohistochemical study was done against these glands. Predominant localization of D-Asp was shown in parotid acinar cells, while D-Asp is specifically located in striated duct cells in submandibular gland. These results suggest that D-Asp may play different and important role(s) in these glands. Furthermore, intraperitonealy administrated D-Asp was absorbed efficiently into these two glands in young, but not in adult. It was indicated that D-Asp accumulation system from blood flow into cell existed in immature rat salivary glands and its system disappeared during growing up.

The presence of high concentrations of free D-amino acids in human gastric juice and saliva

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Free neutral D-amino acids have previously been detected in human plasma from healthy individuals, accounting for less than 2% of the total free amino acid concentration (D-amino acid ratio). It has also been reported that the plasma level of D-amino acids increases up to 10-fold over the normal level, in patients with renal disorders (Nagata Y. et al., Experientia 48 (1992) 986–988). In the present study, in order to determine the source of free D-amino acids, the ratios of the D- and L-enantiomers of the major non-essential amino acids, i.e., the free form of serine, alanine, proline, aspartate and glutamate, in human gastric juice, saliva, the submandibular glands, and oral epithelial cells were analyzed by HPLC. High concentrations of D-alanine and D-proline were detected in saliva and gastric juice. The D-amino acid ratios for alanine and proline were 35% and 20%, respectively, in the saliva, and the maximum values for alanine and proline in gastric juice were 26% and 29%, respectively,. The effects of ingested food and some oral bacteria on the saliva amino acid levels were shown to be insignificant. In the submandibular glands, D-alanine and D-aspartate were detected in ratios up to 5%, and D-alanine and

D-proline were found in oral epithelial cells in ratios of 18% and 5%, respectively.

Alanine racemase from the green alga *Chlamydomonas reinhardtii*

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Chlamydomonas is a genus of unicellular green algae. Although this green alga has recently been used as a model system in many fundamental studies in cell biology and molecular biology, there have been few studies carried out regarding the presence of D-amino acids. And, in the plant, only a few researches regarding the presence of an amino acid racemase has been done. In the present study, we report for the first time the presence of an amino acid racemase in the green alga *Chlamydomonas reinhardtii* and the characteristics of the enzyme.

C. reinhardtii was grown in a phosphate-buffered medium supplemented with 0.1% acetic acid and Hunter's trace elements under constant illumination at 30°C. The cells were disrupted by supersonic treatment and centrifuged. The supernatant was used as a cell free extract. The enzymatic activity of amino acid racemase was assayed with L-alanine, L-serine, and L-proline as substrates. The results indicated the activity for alanine was the highest, and thus alanine was used as the substrate in the subsequent experiments. The enzyme was partially purified by ammonium sulfate fractionation followed by three kinds of HPLC using DEAE Toyopearl, Phenyl Sepharose, and Shodex KW-2003 columns. The enzyme showed a maximum activity at 45°C and pH 9.0. The enzymatic activity was absent in the presence of 20 mM hydroxylamine or 1 mM sodium borohydride. The activity was recovered by adding 0.05 mM PLP. These results indicate that the enzyme requires PLP as a coenzyme.

Determination of D-amino acids in cocoa beans (Theobroma cacao L.) and cocoa products

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Free amino acids (AA) in cocoa products (cocoa powder, chocolate powder, and chocolate) and in cocoa beans (Theobroma cacao L.)

From Sulawesi and the Ivory Coast were analyzed for D-amino acids. Beans were roasted at varying temperature and time. AAs were isolated using Dowex 50W X8 cation exchanger and converted into volatile N(O)-pentafluoropropionyl amino acid (2)-propyl esters. Derivatives were separated by gas chromatography on Chirasil-L-Val® capillary columns and characterized and quantified by selected ion monitoring mass spectrometry (GC-SIM-MS) using L-norleucine as internal standard.

Quantities of D-Ala ranged from 1.1% (fermented raw beans) to 17.6% (beans roasted at 150°C for 120 min). Quantities of Asx and Tyr amounted to 11.1% D-Asx and 17.0% D-Tyr, whereas D-Ser, D-Leu, and D-Val were found as minor components. Thus amounts of D-amino acids increased on roasting but microbial fermentation of raw beans pulpa did not increase the D-AA content of beans. In cocoa products and chocolate rich in cocoa typically 7–12% D-Ala and 5–12% D-Tyr were detected as well as lower quantities of other D-amino acids.

Main reaction in cocoa beans during roasting is the Maillard reaction (non-enzymatic browning) which also leads to flavor and color compounds (melanoidins). It is assumed that D-AAs are released from Amadori compounds (fructose-amino acids) which are abundant in cocoa products.

D- and L-aspartic acid have opposing modulatory actions on L-glutamate induced gating of the ionotropic AMPA-like glutamate receptor SqGluR

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SqGluR is a ionotropic AMAP-like glutamate receptor subunit cloned from the nervous system of the squid (Battaglia et al., 2003). Although L-glutamate is the presumed neurotransmitter at the synapses expressing this receptor, considerable concentrations of D- and L-aspartic acid are also present in cephalopod nervous system and may represent potentially novel neurotransmitters or neuromodulators. With this idea in mind, we examined HEK 293 cells transfected with a construct containing the full length SqGluR clone along with a sequence coding for GFP (Green Fluorescent Protein) (Chun et al., 2005). Transfected HEK cells were voltage clamped and exposed briefly to 10 mM L-glutamate and D and L-aspartic acid. At a holding potential of −60 mV L-glutamate evoked an inward current of around 1 nA. Neither D or L-aspartic acid evoked a current when applied alone. However when 10 mM L-glutamate was added in the presence of 1 mM D- or L-aspartic acid, massive inhibition (in the case of D-aspartic acid) or potentiation (in the case of L-aspartic acid) were observed. These data are the first indication that D and L-aspartic acid are able to directly modulate receptors sensitive to L-glutamate and could indicate that these substances act as neuromodulators of L-glutamate receptors *in vivo*.

Endocrine roles of D-Aspartic acid in the lizard, *Podarcis s. sicula*

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In the lizard *Podarcis s. sicula*, a substantial amount of D-Aspartate (D-Asp) is endogenously contained in the testis where D-Asp is synthesized from L-Asp by an aspartate racemase. The testis of lizard shows cyclic changes of activity connected with reproduction. Our *in vivo* experiments, consisting of i.p. injections of 2.0 μmol/g b.w. of D-Asp in lizards collected during three main differing phases of reproductive cycle (pre-reproductive, reproductive and post-reproductive period) revealed that testis has the ability to take up and accumulate D-Asp. Moreover, this amino acid influences the synthesis of steroid hormones in all phases of the cycle. This phenomenon is particularly evident during the post-reproductive period, when endogenous testosterone levels observed in the both testis and plasma were the lowest and 17ß-estradiol concentrations were the highest, i.p. administration of D-Asp rapidly induces decreasing of 17ß-estradiol and an increasing of testosterone contents. In the pre-reproductive period, when a new set of spermatogonia came into spermatogenesis, the exogenous D-Asp induces a significant increasing on the mitotic activity of the testis. The spermatogenesis induction by D-Asp is recognized by an intense immunoreactivity of the germinal epithelium (SPG and SPD) for proliferation cell nuclear antigen (PCNA). This studies sustains the role for this excitatory amino acid in the steroidogenesis and spermatogenesis in the testis of the lizard *Podarcis s. sicula*. The effect of D-Asp in testis appear to be specific since they where not seen in the lizards injected with other D- or L-amino acid with known excitatory effect on the neurosecretion. In the summarizing

scheme below is shown D-Asp effect on plasma and testicular sex hormones (testosterone and 17ß-estradiol) profiles and on the mitotic activity (immunoreaction for PCNA) during spermatogenesis. Bold arrow indicates the administration of D-Asp (2.0 μmol/g b.w.) to animals and samples (plasma and testis extracts and sections) were obtained 0, 3, 6, 15–24 hrs from D-Asp injection. Hormonal curves indicate a trend only and do not represent quantity. The photos represent immunopositivity for PCNA. SPG, spermatogonia; SPD, spermatids.

Respiration using D-valine in the hyperthermophile *Pyrobaculum islandicum.*

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Pyrobaculum islandicum is a strictly anaerobic archaeon that grows optimally at 100°C. The organism was isolated from boiling neutral solfataric water in Iceland, and grows both organotrophically on complex organic compounds such as peptone, and lithotrophically using H_2 as an electron donor and sulfur as an electron acceptor. We previously reported on the D-amino acid contents and D-amino dehydrogenase activity of *P. islandicum* (Nagata et al., B.B.A 1435 (1999) 160–166), although with respect to respiration, the electron transfer system has not been elucidated in. Research in our laboratory has indicated that D-proline or D-valine reduces a *b*-type cytochrome in *P. islandicum* cell-free extract based on absorption spectra. Further purification of the *b*-type cytochrome was carried out as follows. The cells were disrupted by a French press, and the homogenate was centrifuged to remove unbroken cells. The membrane fraction was separated by ultracentrifugation (140,000 $g \times 60$ min) of the supernatant. D-Amino acid dehydrogenase was obtained by solubilization of the membrane fraction with 0.2% Newcol and sonication. The insoluble fraction was solubilized with 5% Triton X-100. As a result, *b*-type cytochromes with absorption maxima at 559 nm and 557 nm were obtained by ammonium sulfate fractionation and ion exchange chromatography on CM-Toyopearl. The former cytochrome is reduced by the above D-amino acids.

D-aspartate affects secretory activity of the harderian gland

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D-aspartate (D-Asp) is found endogenously within the harderian gland (hg). This is an orbital gland present in many tetrapod species that possess a nictating membrane. Although its main role is to lubricate the eye, several other functions have been suggested, for example: source of pheromones and growth factors; osmoregulation; immunoreactive organ. Our *in vivo* experiments, consisting of i.p. injection of 2.0 μmol/g b.w. D-Asp in both lizards, *Podarcis s. sicula* and frogs, *Rana esculenta*, revealed that hg of both species can take up and accumulate the amino acid. The lizard hg consists of two different parts: the medial zone is mucous acinar gland and the lateral is serous tubulo-acinar gland. Exogenous D-Asp prevalently accumulated in the lateral part, where it induced serous secretion. Following D-Asp injection highly sulphated mucosubstances increased in the medial part of the gland.

The harderian gland of the frog, *Rana esculenta* is a seromucoid gland that display seasonal changes in secretory activity. At the time when the gland shows low secretory activity, exogenous D-Asp rapidly induced activation of ERK1 and an increase in cells active in RNA synthesis. This increase in transcriptional activity was followed by a significant increase in mucous secretion. By contrast, D-Asp administration when hg is showing high activity rapidly induced inhibition of both ERK1 and transcriptional activity. It is possible that in the frog hg, D-Asp mediated NMDA activation may enhance the kinase pathway. The above activation of opposing stimulatory and inhibitory processes could reflect different levels of NMDA-receptor activity, which could vary as a function of the level of frog hg activity.

D-Aspartate oxidase of the yeast *Cryptococcus humicola*: cloning, characterization and physiological role

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D-Aspartate oxidase (DDO) catalyzes the oxidative deamination of acidic D-amino acids. The enzymes are widely distributed in eukaryotes and have been characterized from various organisms. DDO from the yeast *Cryptococcus humicola* (ChDDO) has quite different properties from other DDOs, including much higher specificity to D-aspartate. To investigate the structure-function relationship and the physiological role of ChDDO, we cloned the gene and characterized its expression profile and the gene-disrupted strain. *ChDDO* gene contains an open reading frame of 1,110 bp encoding a protein with common features of DDO and D-amino acid oxidase (DAO). Phylogenetic analysis suggested that ChDDO is more closely related to fungal DAO than to animal DDO. The gene product expressed in *Escherichia coli* possessed essentially the same properties as the native enzyme. In the yeast cells, ChDDO expression was regulated at the transcriptional level and specifically induced by D-aspartate, and the significant induction was still observed even in the presence of both preferred nitrogen and carbon sources. In contrast to the wild-type strain, a *ChDDO* gene-disrupted strain did not grow on D-aspartate and grew considerably more slowly on D-glutamate as the sole nitrogen source than the wild-type strains. Furthermore, the disrupted strain was more sensitive to the growth retardation by acidic D-amino acids than the wild-type strain. These results showed that ChDDO is essential for growth on D-aspartate and is partly involved in the utilization of D-glutamate as a nitrogen source and, in addition, that it is also involved in the detoxification of acidic D-amino acids.

D-Amino acid respiration in *Eschericia coli*

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D-Alanine is ubiquitous among many bacterial cells both bound in the peptidoglycan cell wall and in free form. However, the physiological role of the free form amino acid has not been extensively studied. Since *Escherichia coli* cells can survive and grow on D-alanine, the respiratory chain of this bacterium with respect to D-alanine was investigated. A cell-free extract containing no cytochrome *bd* complex, and another cell-free extract containing D-amino acid dehydrogenase and cytochrome *bd* complex in addition to cytochrome *bo* complex were prepared. Respiration was examined based on reduction of the cytochromes as observed using the absorption spectra and by the O_2-uptake of the cell-free extracts. The results indicated cytochrome *bo* was reduced by NADH but not by D-alanine, and cytochrome *bd* was reduced preferentially by D-alanine. The O_2-uptake activity with D-alanine as the substrate was measured to be 12% of that with NADH. Electron transport from D-alanine to cytochrome *bd* and oxygen was confirmed using a reconstituted system composed of solubilized D-amino acid dehydrogenase, cytochrome *bo* and *bd* complexes, and ubiquinone-10.

D-Amino acid-containing peptides in platypus venom and their isomerase

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The male Australian platypus, *Ornithorhynchus anatinus*, is the only mammal known to bear a venomous spur on each hind limb. It uses these toxic spurs to assert dominance over other males and to ward off potential enemies such as dogs and humans. The venom is not fatal to humans but causes immediate excruciating pain that is not relieved by conventional treatment. The pain is followed by tissue swelling and hyperalgaesia that can last for weeks, and possibly muscle wasting.

The platypus venom contains numerous novel proteins and peptides that warrants further investigation. Among these is a family of four polypeptides of about 5 kDa referred to as defensin-like peptides or DLPs. Although these peptides have no significant sequence similarity with other proteins, their structural fold is very similar to those of the mammalian antimicrobial peptides, β-defensins. The natriuretic peptide OvCNPb from the platypus venom has recently been found to contain a D-amino-acid-residue at the second position of the primary structure. This suggests that other D-amino-acid containing peptides and an interconverting 24nzyme, L-to-D-amino-acid-residue isomerase, might be present in the venom. We are currently focusing our search for D-amino acid containing molecules on DLPs which are the most abundant peptides in platypus venom. Progress is being made in finding and isolating the L-to-D-amino-acid-residue isomerase from the venom.

A sensitive direct HPLC method for determination of *N*-methyl-D- and L-aspartate and *N*-methyl-D- and L-glutamate in biological tissues

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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 associated with neurological disorders such as 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 higher molar absorptivity). The diastereomers formed are separated on an inexpensive reversed phase ODS-Hypersil column with elution by 0.1% TFA/water – 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 5–10 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: foot and mantle of the mollusk *Scapharca broughtonii*), confirming the results of other researchers.

Free D-alanine and alanine racemase in aquatic invertebrates

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Alanine racemase (E.C.5.1.1.1) which catalyzes the interconversion of D- and L-alanine is detected not only in microbes but in several aquatic invertebrates. Marine crustacean and bivalve mollusk species are found to contain a large amount of free D-alanine in their tissues, which are known to be biosynthesized from L-form by alanine racemase. We have already purified the enzyme from the muscle of the black tiger prawn *Penaeus monodon* and obtained cDNA clones encoding alanine racemase from both muscle and hepatopancreas of kuruma prawn *Penaeus* (*Marsupenaeus*) *japonicus* for the first time in animal kingdom. These clones were overexpressed in *E. coli* and isolated to homogeneity. The enzymatic properties of these clones are almost the same as those of *P. monodon* counterpart. There is a considerable homology between the close relatives of the prawn, although there is a small difference of amino acid residues. The primary structure of the prawn enzyme showed low homology to that of bacteria.

During seawater acclimation, D- and L-alanine were accumulated in several crustaceans and bivalve mollusks. Thus, both of them are regarded as one of the major osmolytes responsible for the intracellular isosmotic regulation. In *P. japonicus*, it was confirmed that a large increase of alanine racemase activity occurred just after the molting. Under these conditions, D- and L-alanine contents also elevated simultaneously. These data indicate that D- and L-alanine are positively synthesized through the activation of alanine racemase during the molting.

Synthesis

Oxyamino- and sulfoguanidino containing amino acids. Synthesis and incorporation into physiologically active peptides

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The significance of the guanidine moiety's importance in many biologically active compounds cannot be disputed. Compounds containing carboxamidine functionality have been widely utilized in the area of pharmaceuticals or as tools for investigation biochemical processes. The guanidine moiety often causes significant changes in the biological activity of organic molecules. On the other hand, the intensive development of combinatorial chemical libraries has drawn more attention to the preparation of amino acids with a non-proteinogenic side chain in order to increase the diversity of the resulting compounds.

In this context we have been concerned for several years with the preparation and characterization of unnatural amino acids containing a basic functionality (oxyamino or guanidine group) in the side chain.

In this study we describe the synthesis of a new building blocks: NCan and NsArg, as well as their derivatives – structural analogues of canaline and canavanine.

Next, we studied the possibilities of introducing NCan and NsArg into the molecule of biologically active peptides.

Synthesis of imidazolidinones from dehydropeptides

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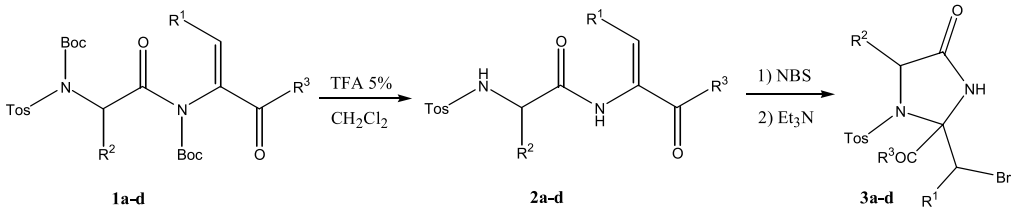
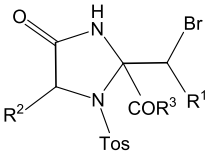
Imidazolidine is an important intermediate and building block in the construction of a variety of biologically active compounds. Its diamine functionality is of great interest in organic chemistry as a chiral auxiliary or metal ligand in catalytic asymmetric synthesis and in medicinal chemistry as a component of various drugs. The hydrophobic nature of imidazolidines can be used to increase the bioavailability of biologically active precursors in the form of a produg.[1]

Dehydrodipeptides obtained by our previously described Boc_2O/DMAP method[2] (compounds **1a**–**d**, Scheme **1**) were treated with a solution of TFA (5%) to remove the *tert*-butoxycarbonyl groups giving the methyl esters of *N*-4-toluenosulfonyldehydropeptides in good to high yields (compounds **2a**–**d**, Scheme **1**). These were reacted with 1.1 equiv. of *N*-bromosuccinimide in dichloromethane followed by treatment with triethylamine to give brominated imidazolidinones in good to high yields (compounds **3a**–**d**, Scheme **1**, Table **1**).



Scheme 1

Table 1. Yields obtained in the synthesis of imidazolidinones from dehydropeptides

Reagent	Product		Yield/%
2a	**3a**	$R_1 = H$, $R_2 = H$, $R_3 = CH_3$	92
2b	**3b**	$R_1 = CH_3$, $R_2 = H$, $R_3 = CH_3$	94
2c	**3c**	$R_1 = CH_3$, $R_2 = CH_3$, $R_3 = CH_3$	74
2d	**3d**	$R_1 = H$, $R_2 = H$, $R_3 = CONHCH_2CO_2CH_3$	82

Compounds **3b** and **3c** were obtained as diastereomeric mixtures. In the case of compound **3c** it was possible to separate the diastereomers by column chromatography.

Defined carriers for synthetic antigens: "Hinge Peptide"

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Using the chemically defined carrier of relatively rigid structure could alleviate the problem with reproduction of effect in the field of synthetic vaccines. Such molecule, represented by parallel hexadecapeptide (H-Thr-Cys-Pro-Pro-Cys-Pro-Ala-Pro-OH)$_2$, has been found in the central part of protein from the surface region of human immunoglobuline. This peptide coordinates four chains of adjacent polypeptides and ensures interactions of immunoglobuline with surroundings and its biological function. We developed syntheses of this peptide and its shortened analogs either in solution or on the soluble (PEG) or insoluble (PS-DVB) polymer supports. In the solution and PEG syntheses, the disulfide bonds were introduced directly to above peptide using S-Trt derivatives of bis-N$^{\alpha}$-Boc-cystine. Thus, the parallel arrangement of both the linear sequences was achieved unequivocally. When using the PEG polymer, the lysine was loaded as the first amino acid to grow the peptide sequence on both the amino groups of this amino acid. Then, an introduction of protected cystine could also be applied. Alternatively to convention mild saponification, the thermolysin was used for detachment of peptide from the PEG in the nearly quantitative yield; however, the C-terminal proline was replaced for enzymatically cleavable leucine. When using the polystyrene support, the Fmoc/tBu, Trt protection was utilized. The disulfide bridges closure by the action of pure oxygen under atmospheric pressure shortened the oxidation time four times in comparison with air oxidation.

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Synthesis and biological activity of RGD derivative with 3,4′,5-trihydroxy-trans-stilbene (resveratrol)*

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Antiplatelet therapy, in general, encompasses all antithrombotic strategies that are based on the inhibition of platelet adhesion and aggregation. It has been demonstrated that several integrins including GPIIb/IIIa, a glycoprotein receptor that is found on platelet surface and is primarily responsible for platelet aggregation, can recognize a common tripeptide motif, the Arg-Gly-Asp (RGD) sequence. Furthermore, red wines have recently been reported to owe their beneficial properties to the presence of resveratrol (3,4′,5-trihydroxy-trans-stilbene), a phytoalexin present in grapes and other food products. Resveratrol has been shown to modulate the metabolism of lipids, and to inhibit the oxidation of low-density lipoproteins and the aggregation of platelets. This compound also possesses anti-inflammatory and anticancer properties.

In the present study, the conjugation of the RGD peptide with resveratrol was attempted, in order to determine whether a more potent inhibitor of platelet aggregation, than the two constituents separately, is obtained. The coupling reaction performed through the protected tripeptide AcArg(Pbf)-Gly-Asp(tBu)-OH, which was synthesized by the solid phase technique using the 2-chlorotrityl chloride resin as a stationary phase. The reaction took place in N,N-dimethylformamide (DMF) with N,N′-dicyclohexylcarbodiimide (DCC) as a coupling reagent. The product was purified by reversed phase HPLC and identified by ES-MS. The characteristic properties as well as the biological activity of the conjugation product against platelet aggregation will be presented.

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Synthesis of new cholic acid containing amphiphiles

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Cell membranes inhibit the entry of many classes of biological active highly hydrophilic and/or charged molecules, e.g. proteins, certain peptides, DNA, oligonucleotides. One way to facilitating the transport of compounds across the biological membranes is to synthesize molecules that mimic the structure of umbrella, i.e. molecules that can cover and attached biological active agent and protect it form an incompatible environment. In this work a cholic acid was used to prepare a bi-walled molecular umbrella. The carboxyl groups of the both steroid units were coupled to the terminal amino groups of the derivatized with ethylene diamine or spermidine glutamic acid. Attachment of the biologically active agent to the remaining amino groups of the glutamic acid yields a molecular umbrella. The model compounds containing dipeptides (Tyr-Arg) and enkephalins as shielded biological agents were obtained. Morphological transformations of model membranes (giant unilamellar vesicles) were investigate in order to elucidate mechanisms of permeabilization co-operating on the level of induced reorganization of the membrane.

Synthesis of 2-amino-oleyl amides and their activity on the enzymes involved in the endocannabinoid system

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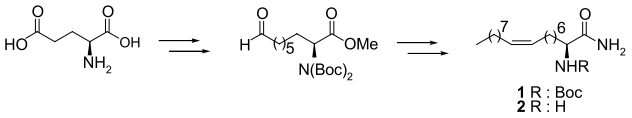
Five proteins involved in the endocannabinoid system, namely CB_1 and CB_2 receptors, fatty acid amidohydrolase (FAAH), 2-monoglyceride lipase (MGL), and anandamide transporter are currently being explored as targets for the development of novel medicinal agents for the treatment of various conditions including pain and inflammation. It has been recently proposed that sterically hindered triacyglycerol analogues are potent inhibitors of digestive lipases. Herein we describe the synthesis of compounds **1** and **2** and the study of their activity on FAAH and MGL. These two compounds are optically active α-substituted analogues of oleyl amide, a natural FAAH substrate. Compounds **1** and **2** were prepared starting from suitably protected glutamic acid. Fully protected L-glutamic acid was reduced into the corresponding γ-semialdehyde and through Wittig-type carbon-chain elongation reactions 2-Boc-amino-oleic acid was synthesized. Saponification, followed by treatment with DCC/HOBt/NH_3 yielded compound **1**. Compound **2** was obtained after



treatment of **1** with 4N HCl. Compound **2** presented a weak inhibition against FAAH, while compound **1** did not exhibit any activity. This result shows the importance of the free amino group. Furthermore, neither of the two oleyl amide analogues tested inhibited MGL.

Acknowledgements

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Synthesis of new 2-pyrrolidinone antihypertensives starting from natural amino acids

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AT_1 antagonists constitute a new generation of drugs, for pressure regulation, with great interest since the stressful life and diet of the modern societies help in the increase of hypertension.

Conformation analysis of AngII and its competitive antagonist [Tyr^4(OMe)]AngII (sarmesin), as well as the AT_1 antagonists belonging to SARTANS class of pharmaceutical molecules, led to the design of new compounds the synthesis of which started from *S*-pyroglutamic or glutamic acid. Some of the synthesized compounds [e.g. (5*S*)-1-benzyl-5-(1H-imidazol-1-ylmethyl)-2-pyrrolidinone], had a considerable bioactivity (>70% compared to the drug losartan). Novel analogues are synthesized as follows:

Methyl (*S*)-pyroglutamate was treated with NaH and subsequently with 1-benzyloxy-3-bromomethyl-benzene or 2-benzyl-5-(2-bromomethyl-phenyl)-2H-tetrazole, and the product was reduced to the corresponding alcohol by $LiBH_4$. The *N*-derivative of (*S*)-pyroglutaminol was activated by conversion to tosylate and was reacted with lithium imidazole. After catalytic hydrogenation the two benzyl type protecting groups of phenolic OH and tetrazole respectively were removed. Another compound was synthesized from *N*-benzyl (*S*)-pyroglutamic acid [which was produced from (*S*)-glutamic acid after *N*-benzylation and heating]. After the introduction of the benzyl group to the enolate position of the ring of (*S*)-pyroglutamic acid by treatment with benzyl bromide and LiHMDS, the carboxylic group was converted to the methyl ester, reduced by $LiBH_4$, and the resulting alcohol was finally converted to the imidazole derivative under conditions already described. The bioactivity of these new analogues is under investigation.

Acknowledgements

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Practical asymmetric synthesis of 6,6-dimethyl lysine derivatives

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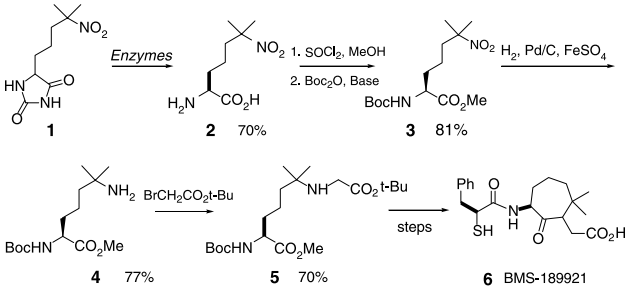
6,6-Dimethyl lysine (**4**) is a key intermediate for the vasopeptidase inhibitor, BMS-189921 (**6**). The syntheses of this compound and its related derivatives have been reported by several groups utilizing the asymmetric hydrogenation of an α, β-unsaturated 6-nitro amino acid, amination to an optically active unsaturated amino acid and the reaction of lithium acetylide with an aziridine carboxylic acid derived from L-Ser.

For several years we have studied the asymmetric syntheses of unnatural amino acids by means of whole cell biotransformation, using microorganisms containing 3 enzymes that effect the enantioselective hydantoin hydrolysis, the enantioselective hydrolysis of N-carbamoyl amino acids and the racemization of hydantoin, respectively. Having screened the appropriate microorganism, the desired amino acid may be theoretically 100% obtainable by this method in either optically active form and in high enantiomeric excess.

We wish to report herein a development of the synthesis 2-*N*-Boc-amino-6-(t-butyloxycarbonylmethylamino)-6-methyl-heptanoic acid methyl ester (**5**). The starting hydantoin (**1**) was readily obtained by the coupling of hydantoin and the aldehyde derived from acrylonitrile (or acrolein) and 2-nitropropane followed by catalytic hydrogenation. The hydantoin **1** was then subjected to hydrolysis by the enzyme to give optical active (S)-2-amino-6-methyl-6-nitroheptanic acid (**2**) in 70% isolated yield. Nitro amino acid **2** thus obtained was esterified and protected by a Boc group to give **3** in 81% yield. The nitro group was successfully converted into an amino group by reduction with Pd-C in the presence of Fe ion. The reaction with t-butyl bromoacetate gave the desired intermediate **5** in 70% yield.



Resolution of chimeric α-hydroxymethyl-α-amino acids derivatives by HPLC on chiral stationary phase

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The interest in enantiomerically pure non-protein α,α-disubstituted amino acids results from their potential as precursors in natural product, peptide and pharmaceutical chemistry. Among the vast family of non-standard amino acids the α-chimeras i.e. bearing two different chains of protein amino acids seem to be very useful in studies of enzyme inhibition, receptor site structure or optimisation of therapeutic activity.

Numerous approaches to optically active α-substituted serine analogues *via* asymmetric syntheses have been reported in the literature. However resolution of easy accessible racemic α-alkylserine derivatives would be superior if efficient procedure is available. HPLC on chiral stationary phase was considered the most promising.

The search for the most convenient racemic substrate involved N-benzoyl-α-alkyl-serines, N-benzoyl-O-acyl-α-alkyl-serines, 4-alkyl-2-phenyl-1,3-oxazol-5-ones and 5-alkyl-5-benzoylamino-4-oxo-1,3-dioxanes. For some N-benzoyl-α-alkylserines and their derivatives

separation of enantiomers could not be achieved or was not satisfactory. The best results have been achieved using S,S-Whelk-O1 stationary phase based on (S,S)-1-(3,5-dinitrobenzamido)-1,2,3,4-tetrahydrophenanthrene as chiral selector. The highest separation factor (ranging from 1.45 to 1.8) has been found for 5-alkyl-5-benzoylamino-4-oxo-1,3-dioxanes – cyclic precursors in racemate synthesis, which easily could be hydrolysed under acidic or basic conditions to α-alkylserines or N-benzoyl-α-alkylserines. For 5-methyl- and 5-benzyl-5-benzoylamino-4-oxo-1,3-dioxanes the order of enantiomers elution has been established. In both cases the faster eluted enantiomer possess (S) configuration.

Calorimetric studies of interactions between N-acetyl-N′-methyl-amino acid amides and urea in water at 298.15 K

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The chemical and biological properties of protein are under the influence of the interaction between this biomolecule and other molecules existing in the environment. The study of those interactions is complicated because of variety of functional groups, as well as a large number of mutually actions of protein and surrounding components. The data of thermodynamic investigations of a simple organic substance containing functional groups analogous to those of protein molecules can be helpful in understanding behaviour of protein in aqueous solutions. By this reason amino acids and small peptides are regarded as a useful model compounds for the study of this type of interactions.

As the objects of the presented work the neutral N-acetyl-N′-methyl-amides of L-α-amino acids and their aqueous solutions of urea as a cosolute have been chosen. The molecules of amides selected for study contain the amino acid side chain close to the peptide groups.

The enthalpies of solutions of N-acetyl-N′-methylglycinamide, N-acetyl-N′-methyl-L-α-alaninamide, N-acetyl-N′-methyl-L-α-leucinamide, N-acetyl-N′-methyl-L-α-serinamide, and N-acetyl-N′-methyl-L-α-threoninamide were measured in water and in aqueous solutions of urea of molality from 0.25 to 3.0 mol(U)/kg(water) using the ''isoperibol'' type calorimeter at 298.15 K. From these data the standard dissolution enthalpies of amides in aqueous urea solutions were determined and the heterogeneous enthalpic interaction coefficients between amino acids derivatives and urea molecule derived from McMillan-Mayer theory have been calculated. The calculated coefficients were compared with the values of enthalpic pair interaction coefficients between analogous amino acids and urea molecule.

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The enthalpic pair interaction coefficients between zwitterions of L-α-amino acids and urea molecule as a hydrophobicity parameter of amino acids side chains

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Natural amino acids, occurring in aqueous solutions in the form of zwitterions ($R-C^{\alpha}HCO_2^-NH_3^+$) as well as the amino acid segments that form peptides and proteins, differ in side chains, –R, from themselves. Side chains differentiate also the interactions of amino acids and their derivatives with surrounding molecules of water and with other molecules or biomolecules occurring in an aqueous medium. Therefore, it is interesting to elucidate the mechanisms and energetics of interactions occurring in aqueous solutions between L-α-amino acids and the substances existing in living organisms or organic compounds which possess analogous functional groups to those in biomolecules. A role of such a model organic molecule is played by urea.

To find the parameters that could differentiate the interactions of amino acid side chains with a molecule of urea in aqueous solutions, the enthalpies of solution of L-α-histidine, L-α-arginine, L-α-proline, L-α-tryptophan, L-α-tyrosine, L-α-lysine, L-α-aspartic acid and L-α-glutamic acid in water and aqueous solutions were measured by calorimetry. The results obtained were used to calculate the enthalpic pair interaction coefficients between zwitterions of amino acids and a molecule of urea in water derived from the modified theory of McMillan Mayer.

The determined enthalpic pair interaction coefficients between the urea molecule and amino acid zwitterions (h_{AU}) describing the summary process of interactions between the examined statistical molecules in solution that takes place with the competitive participation of water molecules well correlate with the hydrophobicity parameter of amino acid side chains found on the basis of the enthalpic homogeneous pair interaction coefficients of natural amino acids.

Therefore, h_{AU} coefficient characterizing side chain amino acids affinity to water can play a role of hydrophobic parameter.

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Thermochemical properties of natural amino acids in ethanol – water mixtures

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The living organisms possess a constant number of free amino acids which participate in all metabolic processes of native cells. Therefore, it is of interest to investigate the interactions in aqueous solutions between amino acid and organic substances that occur in the biological environment or are supplied to it in the form of medicines or cosmetics. Such organic compounds include ethanol as a by-product of glycolysis. Moreover, ethanol is a solvent and a component of numerous pharmaceuticals and cosmetics.

Thermodynamic parameters that characterize the interactions of zwitterions of amino acids with a molecule of ethanol in water solutions include enthalpic heterogeneous pair interaction coefficients, derived from McMillan-Mayer modified theory. These coefficients describe the sum of interactions between ethanol and the amino acids with the competitive participation of water molecules. To determine these parameters, dissolution enthalpies of L-α-alanine, L-α-aminobutiric acid, L-α-valine, L-α-leucine, L-α-isoleucine, L-α-serine, L-α-threonine and L-α-cysteine in aqueous solutions of ethanol were measured by calorimetry at 298.15 K. The obtained enthalpic heterogeneous interaction coefficients between zwitterions of amino acids and ethanol molecule compared with enthalpic heterogeneous interaction coefficients between zwitterions of amino acids and urea molecules.

Lipolanthionine peptides as TLR-2 antagonists – a structure activity relationship

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The N-terminal unusual amino acid S-(2,3-dihydroxypropyl)-cysteine (Dhc), acylated with two or three fatty acids, is found in lipoproteins

from Gram-positive and Gram-negative bacteria and mycoplasma. These lipoproteins and similar synthetic lipopeptides activate cells of the innate immune system *via* Toll-like receptors (TLR-2/TLR-6 and TLR-2/TLR-1). Due to the apparent similarity of the nontoxic scaffolds lanthionine and Dhc, lanthionine-based lipopeptides were synthesized as potential TLR-2 agonists or antagonists. A set of lipolanthionine peptides showed TLR-2 antagonistic activities when applied in a large excess to the agonist $Pam_3Cys\text{-}SK_4$. A detailed structure-activity relationship was performed to investigate the influence of the chirality of the two α-carbon-atoms, the fatty acid (R^1) and fatty amine (R^2) chain length and the oxidation level of the sulphur on the antagonistic activity of the lipolanthionine peptides.

Synthesis of an anthracene-fused 1-aminocyclopentane-1-carboxylic acid (antAib), a novel highly fluorescent $C^{\alpha,\alpha}$-disubstituted glycine

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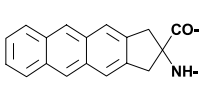
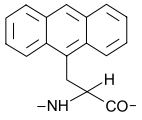
Fluorescence spectroscopy has become a highly valuable technique for conformational studies of biopolymers, the development of peptide-based chemosensors and biochemical research in general. Incorporation of a fluorescent probe into a peptide chain may be achieved by reaction with side-chain functional groups or the direct use of amino acids bearing fluorophores. In this respect synthetic fluorescent amino acids may exhibit significant advantages over protein residues (Trp, Tyr and Phe), in relation with their potentially stronger fluorescence and their more stable excited state. We have designed a new fluorescent aromatic amino acid residue: antAib, which is based on a planar anthracene core and belongs to the class of cyclic, $C^{\alpha,\alpha}$-disubstituted glycines (β-turn and helix inducers in peptides). The antAib residue may be regarded as a rigidified analog of the known antAla residue, but with the spatial disposition of the anthracene side-chain fluorophore relative to the α-carbon atom being completely defined.



antAib



antAla

The synthesis of the terminally protected Boc-antAib-OEt was achieved in seven steps from 1,2,4-trimethylbenzene. Saponification of the ester function afforded the corresponding N-protected amino acid Boc-antAib-OH, suitable for peptide elongation. The fluorescence spectra of Boc-antAib-OH in solvents of different polarities suggest that the antAib residue may represent a useful new reporter group.

Novel synthetic methods for substituted taurines

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During the last two decades aminoalkylphosphonic acid and aminoalkanesulfonic acid derivatives have been widely used as enzyme inhibitors and haptens in the development of catalytic antibodies because of their tetrahedrally structural properties. On the other hand, several 2-aminoalkanesulfonic acids have also been found in many mammalian tissues and they are involved in various and important physiological processes. Thus, it has become very important to develop efficient methods to synthesize structure-diversed aminoalkanesulfonic acids for the investigation on biological activities and the structure-activity relationships. β-Aminoalkanesulfonic acids are very important sulfur analogues of naturally occurring amino acids because α-aminoalkanesulfonic acids and their derivatives are unstable. There are two types of structural analogues of naturally occurring amino acids for β-aminoalkanesulfonic acids, which include 1-substituted taurines and 2-substituted taurines. 2-Substituted taurines have been synthesized effectively *via* sulfite displacement of vicinal amino alcohol methanesulfonates, the peroxy acid oxidation of the thioacetates of vicinal amino primary alcohol, and amino-sulfonation of alkenes. But little attention has been paid to the synthesis of 1-substituted taurines. Herein we describe effective and general methods to the preparation of 1-substituted and 2-substituted taurines from three-membered heterocyclic rings. A series of 2-substituted taurines were prepared *via* sodium sulfite ring-opening of 2-substituted aziridines. A series of 1-substituted taurines were synthesized expeditiously from epoxides either *via* ammonia ring-opening, *N*-protection with benzyl chloroformate, esterification with thiolacetic acid under Mitsunobu conditions, and oxidation with performic acid, or *via* episulfidation with potassium sulfocyanate, ring-opening with dibenzylamine, followed by oxidation with performic acid, and hydrogenolysis in the presence of palladium hydroxide on carbon powder. In our methods, both racemic and optically active substituted taurines were synthesized in good yields.

Synthesis of optically active-cyclopropylalanine and its peptides

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Nonproteinogenic α-methyl-α-amino acids, AA(αMe), are valuable tools for controlling secondary structure in *de novo*-designed peptides. They are known to be $\alpha/3_{10}$-helices inducers, and also to be powerful enzyme inhibitors. There are various routes for synthesis of racemic and enantiomerically pure AA(αMe), but reported methods for the synthesis often need a lot of steps. We have found that various dipeptide derivatives [Z-(S)-Lys(Z)-(R)/(S)-(N-Bzl)AA(αMe)-NH-cHex] (**5**) containing AA(αMe) can be prepared in good yields by the four-component condensation (Ugi reaction) using Z-(S)-Lys(Z) (**1**), benzylamine (Bzl-NH_2) (**2**), alkyl methyl ketone (**3**) and cyclohexyl isocyanide (cHex-NC) (**4**), and that the diastereomers of **5** can be easily separated by the conventional open-column chromatography or thin-layer chromatography. A number of enantiomerically pure α-methyl-α-amino acids (**7**) could be easily obtained by hydrolysis of the separated diastereomers ($\mathbf{5}_{S-S}$ or $\mathbf{5}_{S-R}$), followed by hydrogenolysis. As far as we examined, the S–S diastereomer of **5** elutes faster than the S-R one of **5** not only in the column chromatography using silica gel but also both in TLC and in reversed phase HPLC, without exception.

By the same procedure, we prepared a novel amino acid, (R) and (S)-α-cyclopropylalanine (αCpa). The Ugi reaction using cyclopropylmethylketone as a ketone component afforded a dipeptide derivative containing αCpa residue in a good yield. Diastereomers of the dipeptide were also easily separated by column chromatography. Each diastereomer gave optically active αCpa by acid hydrolysis and then hydrogenolysis. Peptides containing α-Cpa were also prepared, and the conformations of the peptides were studied.

Transport

EAAT3 glutamate transporter is regulated by actin cytoskeleton in C6 rat glioma cells

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In C6 rat glioma cells the transport of glutamate is due to the activity of EAAT3 transporter. In these cells, the incubation with latrunculin A, a toxin that produces a disassembly of actin cytoskeleton, produces a rapid and reversible decrease of glutamate influx, due to a decrease in transport Vmax. Western blot experiments indicated that latrunculin effect was referable to a decrease in the expression of the transporter on the cell membrane. Cytochalasin D, another drug that depolymerizes actin filaments with a distinct mechanism of action, also lowers glutamate transport significantly. The disruption of actin cytoskeleton does not hinder either proline transport (SNAT transporters) or threonine transport (ASCT transporters). Moreover, neither latrunculin nor cytochalasin prevent the increase in glutamate transport caused by phorbol-induced activation of protein kinase C, a regulatory mechanism associated with an increased abundance of EAAT3 protein on the cell membrane. Jasplakinolide, a toxin that stabilizes actin filaments, stimulates the activity of EAAT3 when employed at low ($<1\ \mu$M) but not at high doses. It is concluded that, under basal conditions, the level of organization of actin cytoskeleton influences the membrane trafficking and/or activity of EAAT3 transporter. Moreover, the insensitivity of PKC-dependent stimulation of EAAT3 to actin depolymerization suggests that different pools of EAAT3 transporters exist in C6 cells.

Three-dimensional quantitative structure-activity relationship (3D QSAR) analysis of β-lactam antibiotics and tripeptides as substrates of the mammalian H^+/peptide cotransporter PEPT1

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The mammalian intestinal H^+/peptide symporter PEPT1 displays a broad substrate specificity. PEPT1 transports di- and tripeptides as well as peptide-like drugs such as orally active β-lactam antibiotics and angiotensin converting enzyme inhibitors. The utilization of the transport protein PEPT1 as drug delivery system is a promising strategy to enhance oral bioavailability of drugs. Since very little is known about the substrate binding site of PEPT1 computational methods are a meaningful tool to get a more detailed insight into the structural requirements for substrates. The endogenous substrates of PEPT1 range from the size of Gly-Gly to Trp-Trp-Trp. Therefore, it is necessary to investigate substrates like tripeptides containing voluminous side chains (e.g. Trp-Trp-Trp). Our present 3D QSAR study was performed on a training set of 98 different substrates such as β-lactam antibiotics, di- and tripeptides with respect to their binding affinity towards PEPT1. For this purpose various affinity data of tripeptides and β-lactam antibiotics were determined at Caco-2 cells under identical experimental conditions. A statistically reliable model of sufficiently high predictive power was obtained. The results provided by CoMSIA were graphically interpreted using different field contribution maps. Regions were identified, which are crucial for the interaction between substrates and PEPT1. The model verifies and expands previous models to the full range of compounds known to be substrates of PEPT1. Finally, the 3D QSAR model was applied to design a new, for PEPT1 uncommon drug-like compound mimicking a dipeptide. The predicted K_i value could be confirmed experimentally.

Comparison of OprM and TolC using directed evolution and X-ray structures

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In *E. coli*, the outermembrane protein TolC forms a tripartite complex with the periplasmic adaptor protein AcrA, and the innermembrane protein AcrB. This complex forms an efflux pump that is able to expel a wide range of organic compounds and drugs. This kind of efflux machinery also exists in other gram negative bacteria like for instance *Pseudomonas aeruginosa*. In *Pseudomonas aeruginosa*, the MexAB OprM pump is the main tripartite efflux pump. MexB is the functional homologue of AcrB, while MexA and OprM are the functional homologues of AcrA and TolC, respectively.

On amino acid level there is very little sequence similarity between TolC and OprM. However, as shown by X-ray crystallography, the protein structures of OprM and TolC are highly similar. Despite the high structural similarity between TolC and OprM it has been shown that TolC doesnot interact functionally with MexAB from *Pseudomonas aeruginosa*. In this study we asked three questions:

1 Is it possible to adapt TolC to MexAB?
2 If so, how many amino acid substitutions need to take place in TolC to adapt it to MexAB?
3 In which domains do these changes take place?

In our study we made a TolC mutant library using error-prone PCR followed by DNA shuffling. These mutants were screened in an *E. coli* (*AcrAB TolC*) mutant containing MexAB on a plasmid on LB plates with novobiocin. Transformants that were able to grow on novobiocin, a substrate for the MexAB-OprM system, were selected and the TolC mutants were sequenced and analyzed.

Sequence analysis showed that one to two amino acid substitutions in TolC were sufficient to make it functionally interact with MexAB. All mutations took place in the lower part of the equatorial domain or the tip.

In conclusion, this study shows that there is very little amino acid conservation in the outermembrane proteins. However, despite their huge sequence variation, this study also indicates that the interacting domains of outermembrane proteins are very similar on a structural level and have been largely conserved amongst the outermembrane proteins.

This study indicates that the tip and equatorial domain of TolC and its homologues might be the points of interaction with its periplasmic and innermembrane partners.

Membrane transport of L-proline containing peptides and related drugs

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A promising strategy for the delivery of drugs across cell membranes is the utilization of physiologically occurring transport systems. Consequently, the substrate specificity of carriers, the design of prodrug substrates and the pharmacogenetics/genomics of potential drug transpor-

ters gained enormous interest in recent years. In our group, we focus on the peptide transporter hPEPT1 and the amino acid transporter hPAT1.

hPEPT1 is expressed at the apical membrane of intestinal, renal and biliary duct epithelial cells. The carrier transports di- and tripeptides from the intestinal lumen into the cells in a H^+ symport. L-Proline containing peptides are of particular interest because

(1) Xaa-Pro peptides are very often resistant to enzymatic hydrolysis and display a high affinity to PEPT1. The decisive factor for their interaction with PEPT1 is a *trans* peptide bond conformation. In contrast, Pro-Xaa peptides are often not accepted by PEPT1.
(2) Several orally available angiotensin converting enzyme inhibitors are Xaa-Pro derivatives recognized by PEPT1 because of their sterical resemblance to small peptides.
(3) Side chain modification of Xaa-Pro dipeptides led to the development of the first non-transported, high-affinity inhibitor of PEPT1, Lys[Z(NO_2)]-Pro.
(4) Recently, the most rigid dipeptide analogue Ala-ψ[CS-N]-Pro was used as template for the identification of pharmacophore features and the structural alignment of conformers in 3D-QSAR molecular modeling studies of PEPT1 substrates.

Our results serve as starting points for the characterization of the substrate binding domain of the PEPT1 protein and the development of novel orally available peptidomimetic drugs.

Oligopeptide transporter hPepT1 in intestinal inflammation

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Previously, we have demonstrated that: 1) intracellular uptake of fMLP leads to the activation of inflammatory responses by intestinal cells and 2) the absorbed fMPL ultimately moves across the basolateral membrane to complete the transcellular transport of fMLP. Here, we investigated hPepT1 expression and its ability to transport fMLP in human monocytes. Interestingly, the lamina propria of the human small intestine stained positive for hPepT1, suggesting the presence that hPepT1 is expressed in immune cells. We then examined the hPepT1 expression in the monocytic cell line, KG-1. We demonstrated that hPepT1 is expressed in KG-1 cells. Uptake experiments showed that the transport of 20 μM radiolabeled Gly-Sarcosine (*Gly-Sar) in KG-1 cells was Na^+, Cl^- dependent and DIDS sensitive. In addition, hPepT1 activity was directly coupled to NHE3, as evidenced by the fact that *Gly-Sar uptake was not affected by the absence of Na^+ when cells were incubated in at low pH (5.2). Interestingly, hPepT1-mediated di- and tripeptides were reduced in KG-1 cells incubated atlow pH, likely due to the non-polar nature of KG-1. Finally, we showed that hPepT1 is responsible for transporting fMLP into undifferentiated and differentiated (macrophage-like) KG-1 cells. Together, these results show that hPepT1 is expressed in non-polarized immune cells such as macrophages, where the transporter functions best at the immune physiological level of pH 7.2. Furthermore, we provide evidence for hPepT1-mediated fMLP transport, which might form a novel immune cell activation pathway during intestinal inflammation.

Identification of amino acid residues in the human cationic amino acid transporter hCAT-1 that are crucial for its function

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The human cationic amino acid transporter hCAT-1 exhibits an apparent high substrate affinity and is sensitive to *trans*-stimulation consistent with the classical system y^+. In contrast, hCAT-2A has an apparent low affinity for cationic amino acids and is relatively insensitive to *trans*-stimulation. We have previously shown that chimeras between hCAT-1 and hCAT-2A, where the fourth intracellular loop as well as part of the adjacent transmembrane domain (TM) VIII and TM IX and X (hCAT-2A/1 BK) were exchanged, exhibited the transport properties of the donor of that region. We further demonstrated that the mutation of two amino acid residues within the fourth intracellular loop (R369E and insertion of N at position 381) is sufficient to confer high substrate affinity to hCAT-2A. However, this mutant is still relatively insensitive to trans-stimulation. Interestingly, the reciprocal hCAT-1 mutant (E367R and deletion of N 379) as well as the single mutant (deletion of N379) had virtually no transport activity. This suggested that the adjacent hCAT-1 protein domains need to interact with N379 to build a functional transporter, while the corresponding hCAT-2A domains do not need this interaction. Therefore, in the present study, we introduced small fragments of hCAT-2A into the hCAT-1 single and double mutant (fragment BN containing 12 amino acid residues encompassing parts of TM VIII and the fourth intracellular loop and fragment SK containing TM X). In fact, both mutants gained transport activity. Single back mutations in the BN and SK fragments revealed two and three amino acids residues, respectively, that are necessary to confer function to the deletion mutant. As expected, a hCAT-2A chimera containing the BN and SK fragments of hCAT-1 had almost no activity. Surprisingly, introduction of the BN and SK fragments of hCAT-2A into hCAT-1 lead to transporters exhibiting even lower affinities than hCAT-2A, while the same chimeras with an additional deletion of N379 exhibited apparent K_M values similar to hCAT-1. Our data suggest a complex interaction of TM VIII and X with N379 located in the fourth intracellular loop.

Characterization of vitamin B_1 uptake by BeWo cells, a human placental choriocarcinoma cell line

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Very little is known concerning the mechanisms responsible for the transplacental transfer of vitamin B_1 (thiamine). So, the aim of this work was to characterize the placental uptake of this vitamin from the maternal circulation. For this purpose, we determined the characteristics of ^{3}H-thiamine uptake by a human trophoblast cell line (BeWo), an *in vitro* model of the human placenta. We also aimed at studying the nutritional modulation of the placental uptake of thiamine and in this context we investigated the acute and chronic effects of several dietary bioactive compounds upon ^{3}H-thiamine uptake by BeWo cells.

Uptake of ^{3}H-thiamine (50–100 nM) by BeWo cells was: 1) temperature-dependent and energy-independent; 2) pH-dependent (with increased uptake as the extracellular medium pH decreased); 3) Na^+-dependent and Cl^--independent; 4) not inhibited by thiamine structural analogs (amprolium, oxythiamine and thiamine pyrophosphate); 5) inhibited by the unrelated organic cations guanidine, *N*-methylnicotinamide, tetraethylammonium, clonidine and cimetidine; 6) inhibited by the organic cation serotonin, and by two selective inhibitors of the serotonin plasmalemmal transporter (SERT), fluoxetine and desipramine.

Moreover, uptake of ^{3}H-thiamine (100 nM) by BeWo cells was stimulated by chronic exposure of the cells to the xanthine caffeine (1 μM) and was inhibited by chronic exposure to the polyphenolic compounds xanthohumol and isoxantohumol (at 1 and 0.1 μM, respectively). None of these compounds had an acute effect upon ^{3}H-thiamine uptake by BeWo cells.

In conclusion, ^{3}H-thiamine uptake by BeWo cells seems to occur through a process distinct from ThTr-1 and ThTr-2, two cloned thiamine transporters. Instead, ^{3}H-thiamine uptake by these cells seems to involve SERT, a serotonin transporter. This transport system seems to be non-directly modulated by distinct dietary bioactive compounds.

Mechanisms underlying the upregulation of cysteine uptake by oxidative stress in isolated cardiac myocytes

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Oxidative stress depresses cardiac function and contributes to ischaemia-reperfusion injury of the myocardium. Synthesis of the major cardiac antioxidant glutathione is dependent on the availability of cysteine. The aim of this study was to investigate how cysteine transport into heart cells is affected by oxidative stress.

Isolation of cardiac myocytes and measurement of 0.1 mM [^{35}S]cysteine transport were performed as described previously. Oxidative stress was induced by incubation with 50 μM H_2O_2 at 37°C ± an inhibitor of RNA transcription, actinomycin D (1 μM) or an inhibitor of protein synthesis, cyclohexamide (10 μM). Effects on cell viability were assessed by Trypan Blue exclusion. All results are expressed as means ± S.E.M. and were analysed using ANOVA with a Dunnett post-test.

None of H_2O_2, actinomycin D, or cyclohexamide affected cell viability during a 4-hour incubation. Oxidative stress led to a time dependent increase in the sodium dependent component of cysteine uptake, which became significant after 4 hours. The following table shows the effects of actinomycin D and cyclohexamide on cysteine uptake after 4 hours exposure to oxidative stress.

Condition	Uptake	% of control
Control	83 ± 6.9	100 ± 8.4
$+H_2O_2$	146.9 ± 9.6*	177 ± 11.6*
$+H_2O_2$ & Actinomycin D	98.9 ± 6.9	119.1 ± 8.3
$+H_2O_2$ & cyclohexamide	69.1 ± 9.3	83.3 ± 11.2

* $p < 0.05$ vs. all other conditions.

In conclusion these results suggest that oxidative stress increases cysteine uptake through a mechanism possibly involving more RNA transcription and protein synthesis.

Characterization and photoaffinity labeling of the H^+/peptide cotransporter hPEPT1 heterologously expressed in HRPE cells

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The H^+/peptide symporter hPEPT1 transports small peptides and peptidomimetics such as amino-β-lactam antibiotics across the apical membrane of intestinal epithelial cells. Because of its potential as a drug delivery system there is growing interest in the mechanism, the specificity and the structure of hPEPT1. So far the three dimensional structure of the carrier is unknown. Moreover, not much is known about the substrate binding site.

We expressed hPEPT1 transiently in the membrane of human retinal pigment epithelium (HRPE) cells. HRPE cells were first infected with the recombinant vaccinia virus MVA encoding T7 RNA polymerase and then transfected with the plasmid pBluescript carrying the hPEPT1 gene under control of the T7 promotor. After 16 h transport of [^{14}C]glycylsarcosine was measured. Uptake was stimulated 7-fold by an inwardly directed H^+ gradient. The uptake of Gly-Sar was linear for 30 min and substrate-saturable with a Michaelis-Menten constant of 0.4 mM and a maximal velocity of 2.2 nmol/20 min per 500.000 cells. Unlabeled Gly-Sar, Ala–Ala, δ-aminolevulinic acid, cefadroxil, Ala-4-nitroanilide, Lys[Z(NO_2)]-Pro and many other known substrates but not glycine or sarcosine inhibited [^{14}C]Gly-Sar uptake. The results were very similar to those obtained in Caco-2 cells.

For photoaffinity labeling studies we synthesized and tested the not-transported hPEPT1 inhibitor benzoylphenylalanyl-alanine ($K_i = 32$ μM). After incubation and photoactivation of HRPE-hPEPT1 cells with the inhibitor (100 μM) the [^{14}C]Gly-Sar transport was inhibited by 71%.

These studies serve as a starting point for the molecular characterization of the carrier's substrate binding site.

Molecular cloning and characterisation of the mouse IMINO system

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The SLC6 family consists of transporters for amino acids, neurotransmitters and osmolytes. These transporters play an important role in the removal of neurotransmitters in brain tissue and in amino acid transport in epithelial cells. The SLC6 family also contains a number of orphan transporters. Recently we identified a new member of the SLC6 family (B^0AT1 or SLC6A19), which is closely related to the orphan transporters and transports neutral amino acids. We hypothesized that other orphan transporters may be amino acid transporters as well. To test this hypothesis we studied the mouse SLC6A20 gene. The mouse has two homologues that correspond to the human SLC6A20 gene, which are known as XT3 and XT3s1. RT-PCR analysis revealed expression of XT3s1 in the brain, kidney, small intestine, thymus, spleen and lung, while expression of XT3 was restricted to kidney and lung. Subsequently, we isolated full-length cDNA clones of XT3s1 and XT3 from brain and kidney, respectively. *In situ* hybridisation showed strong expression of XT3/XT3s1 in the proximal tubules of kidney cortex, in intestinal villi and in the brain. Expression of mouse XT3s1, but not XT3, in Xenopus laevis oocytes induced electrogenic Na^+- and Cl^--dependent transport of proline, hydroxyproline, glycinebetaine, MeAIB and pipecolic acid. The substrate specificity and mechanism fit well with the properties of the IMINO system, one of the major proline resorption systems of the intestine and kidney. Together, the expression pattern and functional characteristics of SLC6A20 suggest a possible involvement in the inherited aminoaciduria iminoglycinuria.

Membrane transport of L-proline and related drugs

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The proton-coupled amino acid transporter 1, hPAT1, mainly expressed in human intestine, brain and lung presently receives much attention as a possible system for oral drug delivery. The L-proline uptake in Caco-2 cells is H^+-dependent, Na^+-independent and mediated by a single transport system (hPAT1) with an Michaelis Menten constant of 2.0 ± 0.2 mM. D-Proline, GABA, taurine, trans-4-hydroxy-L-proline, cis-4-hydroxy-L-proline, cis-4-hydroxy-D-proline, 3,4-dehydro-D,L-proline, L-azetidine-2-carboxylic acid, 3-amino-1-propanesulfonic acid, D- and L-pipecolic acid,

L-thiaproline and many others strongly inhibited the uptake of L-[^{3}H]proline. These compounds are not only recognized but also transported by hPAT1 across the membrane. L-Tryptophan and its derivatives tryptamine, 5-hydroxy-L-tryptophan, serotonin and indole-3-propionic acid also strongly inhibited L-[^{3}H]proline uptake with inhibition constants (K_i) of 0.9 to 6.1 mM. Uptake of L-[^{3}H]tryptophan into Caco-2 cells on the other hand was not inhibited by L-proline. When PAT1 was expressed in *Xenopus laevis* oocytes and analyzed by the two-electrode voltage clamp technique, glycine elicited high inward currents that were dependent on membrane potential and inhibited by L-tryptophan, tryptamine and serotonin. No currents were observed with either L-tryptophan, tryptamine, 5-hydroxy-L-tryptophan or serotonin. We conclude that L-tryptophan and certain biogenic amines bind to PAT1, inhibit transport function but are not transported by this carrier protein. They may be considered as the carriers' naturally occurring inhibitors. In several other cell lines of kidney, brain and skin we obtained functional evidence for membrane expression of the recently cloned sodium-coupled imino acid transporter SIT1 but not PAT1.

An ABC-type cysteine transporter in *Campylobacter jejuni* inferred from the structure of a putative extracytoplasmic solute receptor

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Campylobacter jejuni is a Gram-negative food-borne pathogen associated with gastroenteritis in humans as well as cases of the autoimmune disease Guillain-Barré syndrome. *C. jejuni* is asaccharolytic because it lacks an active glycolytic pathway for the use of sugars as a carbon source. This suggests an increased reliance on amino acids as nutrients and indeed the genome sequence of this organism indicates the presence of a number of amino acid uptake systems. Cj0982, also known as CjaA, is a putative extracytoplasmic solute receptor for one such uptake system as well as a major surface antigen and vaccine candidate. The crystal structure of Cj0982 reveals a two-domain protein with electron density in the enclosed cavity between the domains that clearly defines the presence of a bound cysteine ligand. Fluorescence titration experiments were used to demonstrate that Cj0982 binds cysteine tightly and specifically with a K_d of $\sim 10^{-7}$ M consistent with a role as a receptor for a high affinity transporter. These data, supported by comparative genomic analysis imply that Cj0982 is the binding protein component of an ABC-type cysteine transporter system and that cysteine uptake might be important in the physiology of *C. jejuni* and related bacteria.

Alteration of the transport of L-tryptophan into the liver of rats by α-methyl-DL-tryptophan administration

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We have demonstrated that the hepatic uptake of L-trypotophan (Trp) *via* its high-affinity transport system is closely related to the activity of tryptophan 2,3-dioxygenase (TDO) in rats. Treatment of rats with α-methyl-DL-tryptophan (AMT) is known to induce hepatic TDO and Trp depletion and to inhibit hepatic protein synthesis. Therefore, we examined the effect of AMT on the transport of Trp into the liver of rats. Rats administered intraperitoenally with AMT (2 or 10 mg/kg body weight) were sacrificed 16 h later. The hepatic uptake of Trp was measured by the method of liver perfusion in a non-circulating system. All AMT-administered rats had decreases in serum and hepatic Trp concentrations but showed no changes in serum concentrations of free fatty acids, albumin and branched-chain and aromatic amino acids. AMT-administered rats showed no change in hepatic TDO and protein synthetic activities and protein concentration at a low dose but had an increase in that enzyme activity and decreases in hepatic protein synthetic activity and protein concentration at a high dose. When the uptake of Trp into the liver of rats with and without AMT administration *via* a high affinity transport system was examined using 10 μM Trp, the AMT-administered animals had an increase in the Trp uptake activity at a low dose but no change in that activity at a high dose. These results indicate that AMT administered at a dose of 2 mg/kg body weight increases the transport of Trp into the liver of rats by inducing hepatic Trp depletion only.

Transcriptional regulation of CAT expression and function in cultured vascular smooth muscle cells

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Exposure of cultured cells to inflammatory mediators results in the parallel induction of the inducible nitric oxide synthase enzyme (iNOS) and of the cationic amino acid transporters (CATs) associated with the uptake of its substrate, L-arginine. Both processes are dependent on de-novo protein synthesis and may require activation of select transcription factors of which the ubiquitous NF-κB may be essential for iNOS expression. In contrast, relatively little is known about the transcriptional regulation of CAT induction. In view of the critical relevance of enhanced L-arginine transport in inflammation, we have investigated the requirement of two transcription factors, NF-κB and AP-1 on cytokine and endotoxin induced expression of CATs in rat cultured vascular smooth muscle cells (RASMCs). For these studies, confluent monolayers of RASMCs were activated with a combination of bacterial lipopolysaccharide (LPS) and interferon-γ (IFN-γ) in the absence and presence of selective inhibitors of these pathways. In addition, dominant negative IκB was also transfected into cells and NO production was monitored by the Griess assay 24 h after exposure of cells to LPS (100 μg ml^{-1}) and IFN-γ (100 U ml^{-1}). Changes in iNOS expression was determined by western blotting and transport of L-arginine was monitored using L[^{3}H]arginine. Band-shift assays were used to monitor the changes in activation of either NF-κB or AP-1. The results obtained show that dominant negative IκB and inhibitors of NF-κB but not AP-1 blocked or significantly inhibited induced NO synthesis, iNOS expression and arginine transport. Band-shift assays confirmed the activation of both NF-κB and AP-1 with the activation of these transcription factors being blocked by their respective inhibitors. These findings demonstrate that NF-kB and not AP-1 is critical for the induction of both iNOS and CATs under inflammatory conditions.

hPepT1 transports the bacterial proteoglycan-derived muramyl dipeptide, activating NF-κB and production of cytokines in intestinal epithelial Caco2/bbe cells

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Background & Aims: Bacterial proteoglycan-derived muramyl dipeptide (MDP) activates the intracellular NOD2/CARD15 gene product.

How intestinal epithelial cells take up MDP, however, is poorly understood. We hypothesized that the intestinal apical di/tripeptide transporter, hPepT1, transports MDP, thereby activating downstream pathways like NF-κB.

Methods: Time- and concentration-dependent ^{3}H-MDP uptakes were studied in Caco2/bbe (C2) cell monolayers where hPepT1 expression was either over- or underexpressed, using an inducible adenovirus or silencing RNA (siRNA), respectively. NF-κB activation and IL-8 and MCP-1 release were determined by ELISA. NOD2/CARD15 expression was inhibited siRNA. MDP measurements in human duodenal, cecal, and stool samples were performed by partial purification of the N-acetylmuramic acid portion of MDP.

Results: MDP, but not its isoforms (DD or LL) inhibited uptake of the dipeptide glycosylsarcosine (Gly-Sar) in C2 cells, indicating stereoselective and competitive inhibition, with a K_i value of 80 μM. Approximately 90% of the MDP was found in the cytosol, demonstrating uptake rather than binding. The K_m for MDP uptake was 4.3 mM. C2 cells overexpressing hPepT1 demonstrated increased Gly-Sar and MDP uptake, whereas inhibited uptake was observed after siRNA-inhibition of hPepT1. Treatment of C2 cells with MDP activated NF-κB resulting in IL-8 release, an effect blocked by siRNA-inhibited expression of NOD2/CARD15. The MDP content in cecal fluid and stool samples (in normal subjects) was 20–87 μM, contrasting with non-detectable levels in duodenal fluid aspirates.

Conclusion: In colonic epithelial cells, MDP is taken up by hPepT1 and activates NF-κB and chemokine production. Increased hPepT1 expression in chronic colonic inflammation may play an important role in promoting colonocyte participation in host defense and pathogen clearance through increased uptake of MDP.

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Addendum

Expression of antioxidant proteins in hippocampus following 3 days, 3 weeks and 3 months following perinatal asphyxia in the rat

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Background: Perinatal asphyxia (PA) is a major determinant of neurological morbidity in the pediatric population. Animal studies focusing on the subacute sequelae have uncovered a variety of morphological, neurochemical, behavioral and cognitive changes following PA, including deficits of the protein synthetic machinery and aberrant expression of individual brain proteins. The developing brain is uniquely vulnerable to hypoxic-ischemic injury, with a complex evolution of injury that affords opportunities for intervention. This susceptibility of the developing brain to hypoxia among others depends on the presence of antioxidant defense.

Rationale: Reviewing developmental studies, the susceptibility of the fetal brain to hypoxia appears to increase with brain development as gestation approaches term indicating that the status of the anti-oxidant enzymes among other is determining the sensitivity of the developing brain to hypoxia. We therefore decided to systematically study expression-levels of brain proteins in various age groups in control rats and rats that underwent a period of PA, aiming to extend our understanding of the effects of PA on antioxidant protein expression in a time-dependent way.

Methods: Hippocampal tissue of rats with and without PA (at 20 minutes of PA) was dissected from brain at three different time points: 3 days, 3 weeks and 3 months of age, using a proteomic method. Proteins were run on two-dimensional gel electrophoresis with in-gel-digestion and subsequent identification of proteins by MALDI-TOF followed by quantification of protein spots by specific software. 18 antioxidant proteins were identified and quantified.

Results: In 3 days old asphyxiated rats significantly higher expression of thioredoxin peroxidase 1, which turned to significantly reduced expression at 3 weeks and reached normal level at 3 months was observed. Electron transfer flavoprotein alpha-subunit (mitochondrial precursor) was not detected at 3 days but at 3 weeks and 3 months showing comparable expression in both groups, asphyxiated and control. At three weeks the flavoprotein subunit of succinate-ubiquinone reductase was also reduced in asphyxiated rats. At three months peroxiredoxin 6 presented with elevated expression in asphyxiated rats.

Conclusion: PA leads to derangements of antioxidant proteins at different stages of development. Aberrant protein expression may provide evidence for involvement of individual antioxidant proteins in the pathomechanisms of PA. As expression of the majority of antioxidant-proteins remained unchanged, these findings can be considered specific and not simply due to protein derangement by the deficient protein machinery per se.

Searching for hypothetical proteins: Theory and practice based upon original data and literature

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A large part of mammalian proteomes is represented by hypothetical proteins (HP), i.e. proteins predicted from nucleic acid sequences only and protein sequences with unknown function. Data bases are far from being complete and errors are expected.

The legion of HP is awaiting experiments to show their existence at the protein level and subsequent bioinformatic handling in order to assign proteins a tentative function is mandatory.

Two-dimensional gel-electrophoresis with subsequent mass spectrometrical identification of protein spots is an appropriate tool to search for HP in the high-throughput mode. Spots are identified by MS or by MS/MS measurements (MALDI-TOF, MALDI-TOF-TOF) and subsequent software as e.g. Mascot or ProFound. In many cases proteins can thus be unambiguously identified and characterised; if this is not the case, de novo sequencing or Q-TOF analysis is warranted. If the protein is not identified, the sequence is being sent to databases for BLAST searches to determine identities/similarities or homologies to known proteins. If no significant identity to known structures is observed, the protein sequence is examined for the presence of functional domains (databases PROSITE, PRINTS, InterPro, ProDom, Pfam and SMART), subjected to searches for motifs (ELM) and finally protein–protein interaction databases (InterWeaver, STRING) are consulted.

We here provide information about hypothetical proteins in terms of protein chemical analysis, independent of antibody availability and specificity and bioinformatic handling to contribute to the extension/completion of protein databases and include original work on HP in the

brain to illustrate the processes of HP identification and functional assignment.

Proteome analysis of primary neurons and astrocytes from rat cerebellum

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Neurons and astrocytes are predominant cell types in brain and have distinguished morphological and functional features. Although several proteomics studies were carried out on brain, work on individual brain cells is limited. Generating individual proteomes of neurons and astrocytes, however, is mandatory to assign protein expression to cell types rather than to tissues. We aimed to provide maps of rat primary neurons and astrocytes using two-dimensional gel electrophoresis with subsequent in-gel digestion, followed by MALDI-TOF/TOF. 428 protein spots corresponding to 226 individual proteins in neurons and 406 protein spots representing 228 proteins in astrocytes were unambiguously identified. Proteome data include proteins from several cascades differentially expressed in neurons and astrocytes, and specific expressional patterns of antioxidant, signaling, chaperone, cytoskeleton, nucleic acid binding, proteasomal and metabolic proteins are demonstrated. We herein present a reference database of primary rat primary neuron and astrocyte proteomes and provide an analytical tool for these structures. The concomitant expressional patterns of several protein classes are given and potential neuronal and astrocytic marker candidates are presented.