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Endothelium-dependent hyperpolarization of vascular smooth muscle contributes to the vasodilatation evoked by endothelial stimulants. Since the rectifying properties of myo-endothelial gap junctions impede the electrotonic transfer of hyperpolarization from endothelial cells to vascular smooth muscle cells, it is often assumed that a diffusable chemical is involved. Besides NO and prostanoids, endothelial cells produce several compounds capable of inducing hyperpolarization of vascular smooth muscle cell plasma membrane. Thus, during inhibition of the synthesis of both NO and prostaglandins, a component of the endothelium-dependent relaxation to agonists is induced by hyperpolarization and has been attributed to an endothelium-derived hyperpolarizing factor(s) (EDHF).

Carbon monoxide (CO) has been proposed as an EDHF in porcine pulmonary artery based on the inhibition of the relaxation attributed to EDHF by Tin protoporphyrin IX, a heme oxygenase inhibitor. Although the presence of heme oxygenases in endothelial cells is consistent with this idea, the relaxation presumably mediated by EDHF is resistant to oxyhemoglobin (a scavenger of both NO and CO) in canine arteries. H_2O_2 induces hyperpolarization in vascular smooth muscle in porcine coronary artery. However, EDHF-mediated responses are not affected by catalase, a scavenger of H_2O_2 in both porcine and canine coronary arteries.

Anandamide, an endocannabinoid, evokes relaxation in isolated rat mesenteric artery. In this artery, the relaxation attributed to EDHF is impaired by the CB-1 receptor antagonist SR141716A [N-(piperidin-1-yl)-5-(-4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide], the selectivity of which warrants further investigation.

Epoxyeicosatrienoic acids (EETs) induce vascular smooth relaxation by activating large conductance Ca^{2+}-activated K^+ channels. EETs are generated by endothelial cells, through an arachidonic acid epoxygenase pathway(s). Accordingly, in several arteries, cytochrome P450 inhibitors suppress both the release of EETs and endothelium-dependent hyperpolarization and relaxation of vascular smooth muscle attributed to EDHF.

K^+ channels are the targets of EDHF. The involvement of different classes of K^+ channels (e.g. small and large-conductance Ca^{2+}-activated channels) suggests either the existence of several EDHFs, or that EDHF requires a promiscuis transducing mechanism that enables it to interact with different effectors. Thus, the nature of the relationship between EDHF and K^+ channels needs to be assessed further.

Unresolved issues include the lack of specific pharmacological tools to address the physiological roles of the aforementioned candidates as EDHFs. Different K^+ channel blockers affect EDHF-mediated responses depending on species and anatomical origin of the vessel. It is premature to dismiss any of the candidates, since their relationships with K^+ channels are not fully described. In some arteries cytochrome P450 inhibitors are ineffective, in contrast to phospholipase A_2 inhibitors. In canine arteries, this is due to pre-formed EETs that are mobilized by phospholipases. Hence, resistance to P450 inhibitors may not be a sufficient criterion to rule out EETs as EDHF. EDHF has been difficult to detect using cascade bioassays. The poor hydrosolubility of these lipid derivatives could account for this. Indeed, albumin faciliates detection of EDHF using cascade bioassays. However, more studies are needed to assess the compatibility of the metabolism and chemistry of putative candidates with those attributed to EDHF.

447P CERAMIDE/STRESS KINASE-MEDIATED APOPTOSIS

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The endotoxic shock syndrome is characterized by systemic inflammation, multiple organ damage, circulatory collapse and death. Excessive systemic release of tumor necrosis factor (TNF)α and other cytokines purportedly mediates this process. However, the primary tissue target remains unidentified.

The present studies provide evidence that endotoxic shock results from disseminated endothelial apoptosis. Injection of lipopolysaccharide (LPS), and its putative effector TNFα, into C_{57}BL/6 mice induced apoptosis in endothelium of intestine, lung, fat and thymus after 6 hours, preceding non-endothelial tissue damage. LPS or TNFα injection was followed within one hour by tissue generation of the pro-apoptotic lipid, ceramide. TNF-binding protein, which protects against LPS-induced death, blocked LPS-induced ceramide generation and endothelial apoptosis, suggesting that systemic TNF is required for both responses. Acid sphingomyelinase knockout mice displayed defects in LPS-induced endothelial apoptosis and animal death, defining a role for ceramide in mediating the endotoxic response. Further, intravenous injection of basic fibroblast growth factor, which acts as an intravascular survival factor for endothelial cells, protected mice against LPS-induced endothelial apoptosis and animal death.

These investigations demonstrate that LPS induces a disseminated form of endothelial apoptosis, mediated sequentially by TNF and ceramide generation, and suggest that this cascade is mandatory for evolution of the endotoxic syndrome.

448P GLUCOCORTICOID-INDUCED APOPTOSIS: TRANSCRIPTIONAL MECHANISMS

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Glucocorticoids (GC) are widely used for the treatment of leukaemias and lymphomas due to their apoptotic effects on lymphoid cells. GC receptor (GR) is localized in the cytoplasm and is organized in three main domains. The C-terminal region contains the hormone binding domain and the heat-shock protein binding sites. The central basic domain is involved in the DNA-binding activity of the GR. The N-terminal domain is required for the transactivation activity. Upon ligand binding GR is activated and translocates to the nucleus where it binds to DNA specific sequences termed GRE (glucocorticoid response elements) and transactivates downstream genes. GR can also negatively regulates gene expression through protein-protein interaction with transcription factors such as AP-1. Activated GR has been shown to play an important role in GC-induced apoptosis. Results obtained in lymphoid cells suggested the implication of a transactivation pathway involving specific induction of "death genes" in GC-induced cell death. However, others reports demonstrated the implication of a transrepressive pathway, involving repression of "survival genes", like AP- 1, rather than a transcriptional mechanism in GC-induced apoptosis.

CTLL-2 and HT-2 cells are murine lymphocyte cell lines dependent on interleukin-2 (IL2) for their proliferation and survival. In these cells, treatments with RNA or proteins synthesis inhibitors are able to abolish GC-induced apoptosis suggesting that transcriptional mechanisms are involved. RU486, which confers to the GR, repressive activity towards AP-1 but no transcriptional activity, does not induce apoptosis. Aldosterone, which confers to the GR transcriptional activity but no repressive activity towards AP-1, provokes apoptosis of CTLL-2 cells. These results suggest the requirement of transcription by the GR for apoptosis to occur in CTLL-2 cells.

Furthermore IL-2, at saturating concentrations, completely abolishes GC-induced apoptosis in these cells. These results prompted us to assess the influence of IL-2 on GR transcriptional activity in these cells. For this, CTLL-2 cells were transiently transfected with either the MMTV-LUC (mouse mammary tumor virus long terminal repeat driving the luciferase gene and containing 4 GREs) or the GRE-tk-CAT plasmid (one synthetic GRE coupled to the thymidine kinase promoter and driving the chloramphenicol acetyl transferase gene). IL-2 reduces GC-induced MMTV-LUC transactivation but has no effect on GRE-tk-CAT activity, suggesting that a sequence within the composite MMTV promoter could play a crucial role in the inhibitory effect of IL-2.

449P C95(APO-1/FAS)-MEDIATED APOPTOSIS: SIGNALLING AND DISEASE

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APO-1 (Fas/CD95), a member of the tumor necrosis factor (TNF) receptor superfamily induces apoptosis upon receptor oligomerization. The receptor and its ligand are important for apoptosis of peripheral T cells, for downregulation of an immune response and most likely, at least in part, also for peripheral T cell tolerance. In Aids, apoptosis mediated by this system might contribute to the depletion of T helper lymphocytes.
In a search to identify intracellular signalling molecules coupling to oligomerized APO-1 several cytotoxicity-dependent APO-1-associated proteins (CAP) were immunoprecipitated from the apoptosis-sensitive human leukemic T cell line HUT 78 and the lymphoblastoid B cell line SKW6.4. CAP1-3 and CAP4 instantly detectable after crosslinking of APO-1 were only associated with aggregated and not with monomeric APO-1. CAP1 and CAP2 were identified as phosphorylated MORT1 (FADD) [Boldin, M.P., Varfolomeev, E.E., Pancer, Z., Mett, I.L., Camonis, J.H. and Wallach, D. (1995) J. Biol Chem **270**, 7795-7798; Chinnaiyan, A.M., O'Rourke, K., Tewari, M. and Dixit, V.M. (1995) Cell **81**, 505-5122.] . Association of CAP1-4 with APO-1 was not observed with C-terminally truncated non-signalling APO-1. CAP1 and 2 did also not associate with an APO-1 cytoplasmic tail carrying the lpr^{cg} amino acid replacement. Moreover, no APO-1/CAP association was found in the APO-1^{+}, anti-APO-1 resistant pre B cell line Boe. CAP1-4 form a death-inducing signalling complex (DISC) with the APO-1 receptor and are, thus, the first APO-1 associating proteins of a signalling cascade mediating apoptosis.

450P CYTOKINES AND THE CONTROL OF APOPTOSIS IN THE HUMAN ATHEROSCLEROTIC PLAQUE

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Apoptosis is a recognized pathophysiological phenomenon that contributes to the remodeling of human atherosclerotic plaques.

Identification of specific markers for apoptosis in human atheroma is critical to the development of specific therapeutic strategies to control the progression of these lesions. The cysteine protease interleukin-113 converting enzyme (ICE) has been detected in human atheroma, and it has been suggested that ICE may be responsible for apoptotic cell death in human atherosclerotic lesions. However, another cysteine protease, CPP-32 has been reported to be the ICE-like enzyme responsible for the initiation of apoptotic cell death in mammalian cells. This cysteine protease cleaves and inactivates with high efficiency and specificity poly(ADP- ribose) polymerase (PARP), an enzyme required for DNA repair and genome integrity. We studied atherosclerotic plaques from patients who underwent carotid endarterectomy, and analyzed apoptosis by in situ endlabeling of fragmented DNA (TUNEL method) and DNA fragmentation in agarose gel electrophoresis. CPP-32, detected with the use of a specific monoclonal antibody, was highly expressed in almost all atherosclerotic plaques and colocalized with apoptotic cells. Expression of ICE generally paralleled that of CPP-32, but was also detected in plaques negative for CPP32 and showing no apoptosis. Therefore, it is likely that CPP-32 is the effector enzyme responsible for apoptosis in human atherosclerosis, and specific pharmacological modulation of its activity may be of clinical importance in human atherosclerosis.

On the other hand, the development and progression of the atherosclerotic plaque is believed to largely depends on the balance (or imbalance) between locally produced pro- and anti-inflammatory cytokines. Pro-inflammatory cytokines, including TNF and IFNy are promoters of apoptosis, whereas IL-10, an anti-inflammatory cytokine of the Th2 response also abundantly produced by macrophages, prevents apoptosis of macrophages. Using TUNEL method and immunohisto-chemistry we found that IL-10 almost systematically localized to non-apoptotic cells, and the presence of immunoreactive IL-10 was inversely correlated to the occurrence of apoptosis. IL-10 in the plaque appears to be a major endogenous modulator of the apoptotic process in human atherosclerosis.

451P APOPTOSIS AND PROGRAMMED CELL DEATH: A ROLE IN CEREBRAL ISCHAEMIA

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Although physiological cell death has been known for decades, interest in the subject was renewed in 1972 when Kerr, Wyllie and Currie described in detail, the ultrastructural changes characteristic of dying cells and coined the term apoptosis to describe the process. When dying during development or following a toxic insult, cells of different tissue origins display a wide variety of morphological changes. Apoptotic cells undergo shrinkage, nucleus collapses and chromatin is cleaved into nucleosomal fragments. In contrast, necrosis was described as accidental cell death and cells rapidly becomes unable to maintain homeostasis.

A binary classification scheme suggests that physiologically appropriate death is due to apoptosis and that pathological mechanisms involve a necrotic type of death. In this review, we will address developments in our understanding of a potential involvement of apoptotic cell death in pathologies of the CNS, namely following ischemia-induced neuronal death in the rat brain. Recent studies demonstrated: (1) the protection offered by protein synthesis inhibitors; (2) DNA cleavage into oligonucleosome-sized fragments demonstrated by a typical ladder pattern on agarose gel; (3) early endonuclease activation following focal ischemia, as demonstrated by the presence of high molecular weight DNA fragments (300 to 50 kbp) detected by pulsed-field gel electrophoresis; (4) chromatin condensation and apoptotic bodies stained by the TUNEL assay; (5) compact degenerating neurons, exhibiting acute cell shrinkage, a large number of empty vacuoles and aggregation of dense masses of chromatin beneath the intact nuclear membrane, reminiscent of apoptosis Type II (Clarke, 1990).

In summary, most cells that exhibit chromatin condensation and DNA fragmentation are localized at the inner boundary of the infarcted tissue in the penumbra, whereas neurons displaying features of necrotic cell death are mostly located in the ischemic core. Following mild ischemia, wild-type p53 protein exerts a significant and time-dependent effect in the initiation of apoptosis induced via DNA-strand breakage. Subsequently, increased Bax expression was observed in the cytoplasm of dying cells located in the infarct, whereas an increased Bc1-2 staining was detectable in survival cells and reactive glia present at the periphery of the lesion.

These results may indicate that apoptosis contributes to the development of ischemic infarct and during this dynamic process significant procedures on the type of therapeutic interventions in stroke might be envisaged.

452P MECHANISM OF NERVE CELL DEATH IN PARKINSON'S DISEASE

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Parkinson's disease is characterised by a loss of dopaminergic neurons in the substantia nigra. However, not all dopaminergic neurons generate the same extend in this disease. Among several other factors, the neurons which are vulnerable to Parkinson's disease have been shown already in the control mesencephalon to produce a high amount of oxygen free radicals and to be poorly protected against oxidative stress. However, the cause of neuronal loss in the substantia nigra in Parkinson's disease remains unknown. It has been hypothesised that deleterious free radical production might play a role in the death of dopaminergic neurons which ultimate dye by apoptosis. Glial cells may also participate in the mechanisms of nerve cell death by producing cytokines such as tumor necrosis fact α. Indeed, microglial cells producing TNF α are observed in the substantia nigra of patients with Parkinson's disease. Furthermore, dopaminergic neurons in the human substantia nigra express tumor necrosis factor α. In vitro experience on primary mesencephalic cultures of rat embryos show that the activation of this pathway produces apoptosis via the synthesis of oxygen free radicals and the translocation of NF-Kb transcription factor. Post mortem data observed in the parkinsonian mesencephalon also suggests that NF Kb translocation to the nucleus, an index of it activation is observed under the pathological circumstance. These data suggest that this oxidant-mediated apoptogenic transduction pathway may play a role in the mechanism of neuronal death in Parkinson's disease.

453P FROM THE FROG ROOM TO THE RAT MOTO-NEURONE: RECEPTOR MECHANISMS IN CULTURED MOTONEURONES

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In the spinal motoneurone (MN) considerable interaction of neurotransmitters and integration of convergent signals occur. Culturing embryonic MNs with reduced dendritic trees allows easier access to patch electrodes, quantitative pharmacology and a readier investigation of receptor mechanisms. Trophic factors appear to allow survival and maturation of various features present in adult MNs, but these factors are mainly unidentified.

Our long-term cultures of ventral horn neurones from embryonic 14 day (d) rats were enriched by density gradient centrifugation to give >85% cells with ciliary neurotrophic factor (CNTF) MN characteristics. They were grown with ciliary neurotrophic factor (CNTF) on spinal cord glial monolayers for up to 90 d and investigated using patch clamp. The expected profile of MN features includes expression of choline acetyltransferase (ChAT) and calcitonin gene-related peptide (CGRP), voltage-activated currents such as I_A, I_h and I_{Ca}, neurotransmitter-generated currents e.g. to EAAs and 5-HT; possibly also firing properties reflecting neural connections.

Over 85% of neurones were ChAT-positive and a network of CGRP-positive processes was seen. Several types of K current were displayed, including a sustained, outward current (IDR, peak current 6.5±2.4 nA, peak current density 92.1±36.6 pA/pF, means and s.e. means, n=28) and a transient I_A current (peak 4.0±1.2 nA, peak current density 64± 12 pA/pF, n=7). The mixed cation current, I_h, was present in all but the earliest cultures and increased with time in culture. Ih was blocked by 2mM Cs^+ and showed voltage-dependent activation ($V_{0.5}$ -95 ± 8mV, slope-8.6±1.4, n=4). In 12 mM K^+ medium, 5-HT (1-10 μM) potentiated I_h current in 6 cells investigated. Tryptaminergic projections have not developed by d 14 of gestation. It was uncertain whether 5-HT receptors would be expressed. Depolarizations to 1-10 μM 5-HT were initially small or absent, but increased with time in culture up to amaximum of 18mV after 70 d. They were blocked by 0.1 μM ketanserin. 5-HT (1-10 μM) evoked an inward current whose magnitude was positively correlated with culture time ($r = 0.39$, $P<0.01$, mean 147.8±15.3pA, n=48). Most 5-HT-induced current seemed to result from block of a leak conductance and enhancement of a Na^+-sensitive conductance other than I_h; enhancement of I_h may contribute in some cells. Ba^{2+} (2mM) occluded 5HT responses and itself induced an inward current unaltered by time in culture. Synaptic activity with an irregular bursting pattern occurred. Addition of strychnine (10 μM) or bicuculline (30 μM) induced rhythmic patterned bursts, reminiscent of recordings from *in vitro* rat spinal cord and probably triggered by disinhibition.

Coupling mechanism and the expression of peptide receptors have yet to be explored. Staged removal of trophic support shows that MNs will survive without CNTF in medium conditioned by glial cells, contact with which is not necessary. Which features depend for their expression upon synaptic input and/or Ca^{2+} entry have still to be explored.