Abstracts

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A1

Long-Term Effects of an Endothelin-A Receptor Antagonist on Diabetic Cardiomyopathy and Vascular Dysfunction in Diabetic Mice with Myocyte-Specific Overexpression of Endothelial Nitric Oxide Synthase

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Introduction: The roles of nitric oxide (NO) and endothelin-1 (ET-1) in diabetic organ dysfunction are not well understood. We investigated the effects of chronic oral treatment with the endothelin-A (ET_A) receptor antagonist atrasentan on cardiovascular dysfunction in diabetic mice with normal genotype (wild-type, WT) or myocyte-specific overexpression of endothelial nitric oxide (NO) synthase (transgenic, TG). **Methods:** Diabetes was induced with streptozotocin. Four groups of age-matched mice were studied: Untreated WT diabetic (n = 9), untreated TG diabetic (n = 9), atrasentan-treated WT (n = 9), and atrasentan-treated TG diabetic (n = 8). Atrasentan was administered orally via drinking water at 3 mg/kg over 28 days. Hearts were retrogradely perfused at constant flow and myocardial and coronary function determined. **Results:** All mice developed hyperglycaemia without significant differences between groups. Atrasentan treatment improved ventricular function in response to exogenous norepinephrine (3–3000 n*M*), endothelium-dependent coronary microvascular function as well as cardiac oxidant status as evident from reduced tissue malone dialdehyde levels. None of the responses were different between WT and TG hearts. **Conclusion:** The ET_A receptor antagonist atrasentan ameliorated myocardial and coronary function and improved tissue oxidant status, whereas raising myocardial NO levels had neither beneficial nor deleterious effects on diabetic cardiomyopathy in this transgenic model.

A2

Pharmacological Properties of $GABA_A$ Receptors Containing gamma1 Subunits

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$GABA_A$ receptors composed of α_1, β_2 and γ_1 subunits exhibit a restricted expression pattern in the mammalian CNS (e.g. amygdala, pallidum, septum, substantia nigra, thalamus), and thus represent interesting drug targets. Their pharmacological profile, however, is largely unknown. In the present study $\alpha_1\beta_2\gamma_1$ receptors were expressed in *Xenopus* oocytes. Their modulation by 21 ligands (1 μ*M*) from 12 chemical distinct classes was investigated making use of a fast perfusion system. Modulation of GABA-induced chloride currents (I_{GABA}) was analysed with two-microelectrode-voltage-clamp at a GABA EC_{5-10}. Triazolam, Clotiazepam, Midazolam and CGS20625 were most potent and their action therefore studied on $\alpha_1\beta_2\gamma_1$ and $\alpha_1\beta_2\gamma_{2S}$ receptors in more detail. Triazolam displayed the highest potency for $\alpha_1\beta_2\gamma_1$ receptors ($EC_{50}(\alpha_1\beta_2\gamma_1)$ = 92 ± 2 n*M* vs. $EC_{50}(\alpha_1\beta_2\gamma_{2S})$ = 22 ± 3 n*M*) and induced a maximum enhancement of 85 ± 7%, clotiazepam and midazolam enhanced I_{GABA} by 172 ± 24% and 92 ± 8%, respectively, but exhibited 9- and 8-fold reduced potencies ($EC_{50}(\alpha_1\beta_2\gamma_1)$ = 24 μ*M* vs. $EC_{50}(\alpha_1\beta_2\gamma_{2S})$ = 11 μ*M*). The pyrazolopyridine CGS20625 was similar efficient on $\alpha_1\beta_2\gamma_1$ and $\alpha_1\beta_2\gamma_{2S}$ receptors, but displayed a 2-fold higher potency on $\alpha_1\beta_2\gamma_{2S}$ receptors. Our study provides new insight into the pharmacological properties of $GABA_A$ receptors comprising γ_1 subunits and is likely to aid design of specific ligands for this receptor subtype.

A3

Automated Drug Screening of Ligand- and Voltage-Gated Ion Channels on *Xenopus* Oocytes

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We present a novel perfusion system for automated drug screening on ion channels expressed in *Xenopus* oocytes. Drugs are applied to microperfusion chamber (a 15 µl bath covered by a glass plate) by means of a TECAN Miniprep 60. Two inlet channels in the glass cover enable access of two microelectrodes to the oocyte for two electrode voltage clamp. A funnel for drug application surrounds the access channels for the two microelectrodes. Estimated mean time of solution exchange ($t_{10–90\%}$) is about 150 ms. After an initial fast perfusion step (drug application) the chamber is continuously perfused at a slower rate (usually at 1 µl/second). A custom made software permits automation of the drug screening process. Only small amounts of test solutions (about 100 µl) are required for drug screening. Examples are given to illustrate the use of the robot system for screening on hERG channels, $GABA_A$ receptors and nicotinic acetylcholine receptors.

A4

Expression of Prostaglandin D_2 Receptors in Mouse Spinal Cord

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Prostaglandin (PG) D_2 is the most abundant PG in CNS. In addition to its well known effects on sleep and temperature regulation, PGD_2 seems to be involved in the development as well as in attenuation of allodynia. PGD_2 binds and activates two distinct receptors, DP and CRTH2. While the localization of DP receptors has been investigated previously in lumbar spinal cord, CRTH2 expression was detected by Northern blot; its localization, however, is not known to date. The aim of the study was, therefore, (1) to compare the localization of the two PGD_2 receptors in mouse lumbar spinal cord and (2) if the spinal expression of these receptors is influenced by endotoxin treatment. CRTH2-like immunoreactivity was detected in cells throughout the dorsal horn and especially in motorneurons in the ventral horn. The distribution pattern of CRTH2 thereby closely resembled the pattern we observed with DP receptor. Endotoxin treatment of mice caused a long lasting increase of spinal DP as well as a delayed increase in CRTH2 mRNA expression. The results show that the localization of DP as well as CRTH2 receptors seems to be restricted mainly to neuronal cells located in ventral and dorsal horn, thus raising the possibility that spinal PGD_2 can influence efferent as well as afferent transmission. The delayed and long lasting upregulation of spinal PGD_2 receptors after endotoxin treatment may be seen as an indication for the involvement of spinal PGD_2 in the late phase of inflammation.

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A5

Spatial Relationship Between the Selectivity Filter and the Cytoplasmic Vestibule of the Voltage-Gated Na^+ Channel

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A conformational change of the cytoplasmic vestibule of the voltage-gated Na channel gives rise to ultra-slow inactivation (Ius). Ius occurs by an interaction between the selectivity filter (SF) and the adjacent S6 segments, which form the cytoplasmic vestibule. Mutations in the SF, which increase the permeability to K^+, enhance Ius. Hence, an increase in the diameter of the SF may result in an interaction with the adjacent S6 segment, thereby producing Ius. Consequently, Ius should be enhanced by permeation of large cations. The mutation K1237E in $rNa_V1.4$ allows permeation of both K^+ and large organic cations. In K1237E channels we examined the time course of recovery from inactivation produced by a 300 s prepulse to −20 mV. When Na^+ was permeating, 80% of the channels recovered with a time constant of 120 s, characteristic for Ius. During permeation with the large cation choline 100 % of the channels recovered from Ius, indicating that residency of choline in the channel increased Ius. The mutation I1575A in domain IV S6 abolished the modulation of Ius by choline. Therefore, Ius may occur by a widening of the SF giving rise to an interaction with I1575. Thus, the SF forms the roof of the inner vestibule at the level of I1575.

A6

TRPC3 and NCX1 Form a Signaling Partnership in Rat Cardiac Myocytes

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Cation channel proteins of the classical transient receptor potential protein (TRPC) family are considered as pivotal components of phospholipase C-dependent Ca^{2+} signaling in mammalian tissues. Recently, evidence of a physical and functional coupling of TRPC3 to the cardiac type Na^+/Ca^{2+} exchanger, NCX1 was obtained in the HEK expression system. Here, we tested the hypothesis of a signaling complex between TRPC3 and NCX1 in cardiac ventricular myocytes isolated from rat hearts. Endogenous expression of TRPC3 and NCX1 was detected by RT-PCR and Western blotting. Co-immunoprecipitation and GST-

pulldown experiments were used as binding assays and revealed a physical association of TRPC3 with NCX1. Further, fluorescent measurements of intracellular Ca^{2+} demonstrated an NCX-mediated Ca^{2+} influx which is dependent on a Na^+ entry mechanism governed by activated TRPC3 channels. These data indicate TRPC3 and NCX1 as interactive signaling partners which play a crucial role in the Ca^{2+} homeostasis of rat cardiomyocytes.

A7

Skeletal Muscle Cells Adopt Cardiac-Like Sodium Current Properties in a Cardiac Cell Environment

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Objective: Electrophysiological adaptations of skeletal muscle cells in a cardiac cell environment were investigated. **Methods:** An in vitro system was designed to simulate a cardiac cell environment for cultured skeletal muscle cells. Therefore, these were treated with differentiation medium preconditioned by cardiocytes, and their functional sodium current properties were then compared with those of control cells. **Results:** Treatment of skeletal muscle cells with medium preconditioned by cardiocytes significantly altered activation and inactivation properties of sodium currents from "skeletal muscle"- to more "cardiac"-like ones. Sodium currents of cardiac-conditioned cells showed a reduced sensitivity to block by tetrodotoxin. This finding and reverse transcription PCR experiments suggest that a relative up-regulation of the expression of the cardiac sodium channel isoform $Na_V1.5$ versus the skeletal muscle isoform $Na_V1.4$ is responsible for the observed changes in sodium current function. **Conclusion:** Cardiocytes alter the sodium current properties of skeletal muscle cells via a paracrine mechanism. Thereby, skeletal muscle cells with sodium current properties more similar to those of cardiocytes are generated.

A8

Clarifying the Role of Stat5 in Lymphoid Development and Abelson-Induced Transformation

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The Stat5 transcription factors Stat5a and Stat5b have been implicated in lymphoid development and transformation. Most studies have employed Stat5a/b-deficient mice where gene targeting disrupted the first protein-coding exon resulting in the expression of a N-terminally truncated form of Stat5a/b ($Stat5a/b^{\Delta N/\Delta N}$ mice). We have now re-analyzed lymphoid development in $Stat5a/b^{null/null}$ mice having a complete deletion of the Stat5a/b gene locus. The few surviving $Stat5a/b^{null/null}$ mice lacked $CD8^+$ T lymphocytes. A massive reduction of $CD8^+$ T cells was also found in $Stat5a/b^{fl/fl}$ lck-cre transgenic animals. While $\gamma\delta TCR^+$ cells were expressed at normal levels in $Stat5a/b^{\Delta N/\Delta N}$ mice, they were completely absent in $Stat5a/b^{null/null}$ animals. Moreover, B cell maturation was abrogated at the pre-pro-B cell stage in $Stat5a/b^{null/null}$ mice whereas $Stat5a/b^{\Delta N/\Delta N}$ B lymphoid cells developed to the early pro B cell stage. In vitro assays using fetal liver cell cultures confirmed this observation. Most strikingly, $Stat5a/b^{null/null}$ cells were resistant to transformation and leukemia development induced by Abelson oncogenes, whereas $Stat5a/b^{\Delta N/\Delta N}$ derived cells readily transformed. These findings show distinct lymphoid defects for $Stat5a/b^{\Delta N/\Delta N}$ and $Stat5a/b^{null/null}$ mice and define a novel functional role for the N-terminus of Stat5a/b in B lymphoid transformation.

A9

Loss of Tyk2 Accelerates Lymphoma Formation in Eμ-Myc Transgenic Mice

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It is not known, if immunological surveillance plays a role in driving the evolution of "second hits". To address this issue we relaxed immunological control by crossing the Eμ-Myc mice into a Tyk2 deficient background. In control animals (Eμ-Myc/$Tyk2^{+/-}$), we observed the following associations: Loss of p53 with reduced surface immunoglobulin M (sIgM) expression and accelerated disease; overexpression of Bcl-2 with a delayed onset of disease and high sIgM (i.e. high differentiation grade); low sIgM levels and high incidence of liver metastasis. In contrast, Eμ-Myc/$Tyk2^{-/-}$ mice succumbed rapidly to their disease and the loss of p53 or the overexpression of Bcl-2 were irrelevant to the course of the disease. These observations show that the immune system sculpts the evolving tumors. Interestingly, Tyk2 deficiency protects against the infiltration of portal fields by tumor cells.

A10

A Two-Step Model for ER-to-Golgi Trafficking of the GABA Transporter 1

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ER export is mediated by COPII coated vesicles. In mammalian cells these vesicles have been shown to fuse and generate the intermediate compartment (ERGIC). The nature of this compartment and the mechanism of ERGIC-to-Golgi transport have been intensely debated. According to the maturation hypothesis, ERGIC membranes fuse and generate the cis-Golgi network. Here, cargo is transported passively from ERGIC to the Golgi. According to the stationary compartment hypothesis, the ERGIC is a real compartment. In this model COPI components were suggested to control ERGIC-to-Golgi transport. We show that the transmembrane protein GABA transporter 1 (GAT1) recruits the COPII component Sec24D via a motif in its cytosolic C-terminus. In the absence of this interaction, GAT1 leaves the ER in a non-concentrative manner. We also identified the binding site in Sec24D. After exit from the ER, GAT1 is transported to the ERGIC. Here, its export is controlled by a distinct, trihydrophobic motif in the C-terminus. Mutation of this motif to serines (GAT1-SSS) leads to retention in the ERGIC. This is supported by the finding the GAT1-SSS colocalizes with the dominant negative Rab1a (Rab1a-N124I). This compartment is discontinuous with the ER because: (i) using fluorescence recovery after photobleaching we found that GAT1-SSS doesn't recover 90 s after bleaching. (ii) GAT1-SSS leaves the ER (determined by an in vitro budding assay). We propose a two-step model for the ER-to-Golgi transport of GAT1. First COPII mediates cargo concentration and export to the ERGIC. A second motif controls export from the ERGIC to the Golgi transport. Thus our data strongly support the stationary compartment hypothesis.

A11

Selective Kallikrein Inhibitors Attenuate Haemorrhagic Laesions Caused by Kinin Antagonists in Experimental Acute Pancreatitis

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Kinin B_2 antagonists prevent oedema formation in caerulein-induced pancreatitis but at the same time cause haemorrhagic lesions. We have investigated whether this is due to a reduced influx of endogenous protease inhibitors and a concomitant augmentation of tissue kallikrein activity. Pancreatitis was induced in anaesthetized rats by i.v. infusion of caerulein. The animals were pretreated with the B_2 antagonist icatibant and/or selective inhibitors of tissue kallikrein (tKI) and plasma kallikrein (pKI) [Evans et al., Immunopharmacology 1996; 32: 115–116, 117–118]. Tissue samples were excised at 6 h, assessed histologically, and analyzed for hemoglobin. Icatibant inhibited oedema formation but produced an about 10-fold elevation ($p<0.01$) in tissue hemoglobin levels. Although tKI also inhibited the oedema, signs of vascular damage were absent. Haemorrhage induced by icatibant was largely attenuated by a combined treatment with tKI and pKI ($p<0.05$). Influx of endogenous inhibitors of kallikreins was significantly ($p<0.05$) reduced by icatibant and tKI. However, tissue kallikrein activity was increased 10–100 fold by icatibant ($p<0.01$), whereas it was strongly inhibited ($p<0.05$) by tKI. Increased levels of active kallikrein in the pancreas in the absence of oedema formation cause haemorrhagic lesions in this model. Inhibition of tissue and plasma kallikrein thus seems to be a highly interesting strategy for the prevention of this kind of tissue damage in acute pancreatitis.

A12

Prevention by Lamotrigine of Excitotoxic Neural Death in the Rat Brain After Perinatal Asphyxia

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Perinatal asphyxia induces neural injury, which is related to the toxic action of excitatory amino acids. The damaged neurons exhibit features of apoptosis, necrosis or hybrid forms with mixed features of both types of degeneration. The aim of our study was to investigate the possible neuroprotective action of the antagonist of the excitatory aminoacids Lamotrigine (Lam). In 7-day old rats we produced ischemic-hypoxic injury to the left cerebral hemisphere by left common carotid artery ligation followed by a 1-hour exposure to hypoxia. One group of rats (n = 10) received i.p. Lam at 10 mg/kg and a second group Lam at 20 mg/kg. A third group (n = 10) received saline i.p. (control group). The severity of damage was assessed, 7 days after the insult (14 day of life), in the CA1, CA3, CA4 regions of the hippocampus and the dentate gyrus (d.g.) by using light and electron microscopy. The parameter used for the evaluation of the effect of Lam was the total percentage of damaged neurons. Lam at 10 mg/kg reduced both ischemic and necrotic pyramidal neurons of the CA1 region ($p<0.041$). No effect was found in the other regions. At 20 mg/kg Lam provided in CA1 greater protection ($p<0.025$) but also reduced significantly the damage in CA3 ($p<0.036$) and in the d.g. ($p<0.044$). Both doses had no effect in the CA4 region. In conclusion, Lam, especially at high doses, has neuroprotective effects in an experimental model of perinatal asphyxia in rats.

A13

Delineation of Myotoxicity Induced by HMG-CoA-Reductase-Inhibitors in Human Skeletal Muscle Cells

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The 3-Hydroxy-3-methyl-glutaryl-CoA reductase inhibitors (statins) are widely used and well tolerated cholesterol-lowering drugs. In rare cases side effects occur in skeletal muscle, including myositis or even rhabdomyolysis. However, the molecular mechanisms that lead to these muscle-specific side effects are not well understood. Here we show that statins cause apoptosis in differentiated human skeletal muscle cells. The prototypical representative of statins, simvastatin, triggered sustained intracellular Ca^{2+} -transients leading to calpain activation. Intracellular chelation of Ca^{2+} completely abrogated cell death. Moreover, ryanodine also completely prevented the simvastatin induced calpain activation. Downstream of the calpain activation simvastatin led to a translocation of Bax to mitochondria in a caspase 8 independent manner. Consecutive activation of caspase 9 and 3 executed apoptotic cell death which was in part reversed by the co-administration of mevalonic acid. These data delineate the signalling cascade that leads to muscle injury caused by statins. Our observations have implications for improving the safety of this important medication, and explain why physical exercise aggravates skeletal muscle side effects to some extent.

A14

Effects of Cyclooxygenase Inhibition in Human Monocytes on Endotoxin-Induced TNF-α and Cyclooxygenase-2 Expression

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There are several indications that anti-inflammatory drugs which inhibit cyclooxygenase (COX) might in fact stimulate tumor necrosis factor (TNF)-α biosynthesis. Secondly, it has been suggested that prostaglandin (PG)E_2 can enhance the expression of inducible COX-2, thus facilitating its own biosynthesis in inflamed tissue. In this study human endotoxin-stimulated adherent monocytes were used in order to determine whether or not NSAIDs influence COX-2 and/or TNF-α expression within the range of inhibitor concentrations that are required to suppress PG biosynthesis. While it was found that exogenous PGE_2 inhibited endotoxin-induced TNF-α mRNA and protein expression (IC_{50} < 5 n*M*), no effect on COX-2 mRNA expression was observed at concentrations up to 1 μ*M*. Acetylsalicylic acid as well as indomethacin, caused concentration-dependent inhibition of PGE_2 biosynthesis and, at concentrations causing near-complete inhibition, enhanced TNF-α mRNA and protein expression without influencing COX-2 mRNA expression. In addition, both NSAIDs counteracted dexamethasone-induced inhibition of TNF-α biosynthesis, thereby exhibiting an effect opposite to that of exogenous PGE_2. These results suggests that in human endotoxin-stimulated monocytes, NSAIDs by inhibiting COX and thereby decreasing PGE_2 can enhance TNF-α biosynthesis. The facilitation of TNF-α biosynthesis is detectable also in the presence of dexamethasone, and may thereby interfere with its ability to suppress TNF-α biosynthesis in inflammatory disease.

A15

The Role of Tyk2 in Adaptive Immunity

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The mechanisms underlying immunological tumour surveillance are not fully understood. We showed previously that animals deficient for the Janus kinase Tyk2, which are phenotypically normal unless exposed to excessive virus load, are more susceptible to tumours induced by the Abelson oncogene. This effect is due to impaired tumour surveillance in $Tyk2^{-/-}$ mice and NK cells were shown to be important mediators therein. Given the role of Tyk2 in type I interferon- (IFN-α,β) and type II IFN (IFN-γ) as well as interleukin-12-dependent signalling, one might assume that $Tyk2^{-/-}$ animals also display an impaired adaptive immune response. We therefore tested $Tyk2^{-/-}$ mice for defects in T cell cytotoxicity and observed a strong reduction in the capability of $Tyk2^{-/-}$ cytotoxic T lymphocytes to kill target cells in vivo. Cellular adaptive immunity relies mainly on the activation of professional antigen presenting cells such as dendritic cells, their ability to prime T cells and finally the effector function of T cells. The defect of T cell function that we observed might therefore result from a defect in one or several phases of this process. We are currently investigating which cellular compartment is affected and, subsequently, which step(s) in this chain of events is (are) impaired.

A16

A_{2A}-Receptor Signalling in SH-SY5Y Neuroblastoma Cells Is Contingent upon the Action of Retinoic Acid

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Neuroblastoma cells derived from the SH-SY5Y clone can be differentiated into a neuronal phenotype by retinoic acid. The most obvious sign of differentiation, sprouting of

neurites, is maximized by the addition of neurotrophins; the trkB-receptor is induced by retinoic acid receptor transactivation. SH-SY5Y cells express the A_{2A}-adenosine receptor activation of which enhanced neurite outgrowth. Similarly to what has been observed in CNS neurons, SH-SY5Y cells targeted the A_{2A}-receptor to neurite extensions and receptor activation facilitated neurotransmitter release. We also report that in SH-SY5Y cells retinoic acid strongly increased A_{2A}-adenosine receptor mediated cAMP production. Retinoic acid regulated G-proteins and adenylyl cyclases of which all known isoforms were detected by rt-PCR. However, there was no alteration in the receptor expression level; we can also rule out that down-regulation of phosphodiesterase isoforms led to an increase in cAMP. Retinoic acid increased the abundance of $G\alpha_s$ by about two-fold, with no changes in $G\alpha$ or $G\beta\gamma$. Semi-quantitative measurement of adenylyl cyclase mRNA indicated that retinoic acid induced increases in three isoforms (AC IV, V, II). This was consistent with the regulatory pattern of adenylyl cyclase activity in isolated cell membranes. We thus speculate that retinoic acid is a prerequisite for nerve cell activity of adenosine or possible other substances that activate stimulatory receptors.

A17

The Conserved Glutamate (E^{136}) in TM2 of the Serotonin Transporter Is Required for the Conformational Switch in the Transport Cycle

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The alternate access model provides the theoretical framework for understanding how transporters translocate hydrophilic substrates across the lipid bilayer. The model postulates at least two conformations of a transporter, an outward and an inward facing conformation, which seal the translocation pathway to the interior and the exterior of the cell, respectively; there may be a third state, the locked state, where both, access from outside and from within the cell, is occluded. It is not clear, how the conformational switch is triggered in neurotransmitter:sodium symporters (NSS), but Na^+ is likely to play an essential role. Here, we focused on E^{136} of the serotonin transporter (SERT); this residue is conserved in transmembrane segment 2 of NSS and related proteins. Three substitutions were introduced resulting in SERT-E136D, SERT-E136Q, SERT-E136A, which were all correctly inserted into the plasma membrane. SERT-E136Q and SERT-E136A failed to mediate substrate influx into cells, while SERT-E136D did so at a reduced rate. Binding experiments with the inhibitor [^{3}H]β-CIT supported the conjecture that the mutated transporters preferentially adopted the inward facing conformation: [^{3}H]β-CIT interacted with SERT in a manner consistent with binding to the outward facing state. Accordingly, the Na^+-induced acceleration of [^{3}H]β-CIT association was most pronounced in wild type SERT > SERT-E136D > SERT-E136Q > SERT-E136A. Similarly, SERT-E136Q supported substrate efflux in a manner indistinguishable from wild type SERT, while SERT-E136A was inactive. Thus, in the absence of E^{136}, the conformational equilibrium of SERT is shifted to the inward facing (SERT-E136D, SERT-E136Q) or locked conformation (SERT-E136A).

A18

Molecular Bases of Metabotropic Glutamate Receptor 1 Transcriptional Regulation

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Expression of metabotropic glutamate receptor 1 (mGlu1) is restricted to neurons and undergoes dramatic changes during development, in response to environmental modifications and pathological contexts. The mGlu1 gene (GRM1) gives rise to several splice variants, which have a largely different cell-specific expression pattern. The complex mechanisms resulting in the cell-specific expression of mGlu1 and of its splice variants are largely dependent on its genomic structure. Therefore, we have determined the exon/intron arrangement of human, mouse and rat GRM1. By means of 5'-Random Amplification of cDNA Ends (5'-RACE) we have characterized the 5'-end of human and mouse GRM1 and showed that transcription initiation takes place on multiple alternatively spliced first exons. Differential distribution of the alternative mRNA forms starting within each 5'-alternative exon has been demonstrated in several brain areas by RT-PCR. Identification of the core promoter regions and of functional transcription factor responsive elements is presently ongoing, by means of both bioinformatic sequence analysis and reporter gene assays. In this study, we have elucidated the genomic structure of a glutamate receptor, known to play important roles in motor coordination and neuronal plasticity.

A19

The Importance of Helix 8 in G-Protein Coupling and Surface Expression of the A_1-Adenosine Receptor

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The intracellular domain of a G-protein-coupled receptor has four peptide loops. Loop 4 is part of the receptor carboxyl-terminal (c-)tail; in the A_1-adenosine receptor, loop 4 likely folds into an amphipathic helix (helix 8) that extends parallel to the membrane suface. It has been suggested that residues of a conserved motif (NPXXY) present in the 7th transmembrane span stabilize the membrane-oriented side chains of helix 8 and inform the

c-tail on the activity state of the receptor. Mutation of the putatively interacting amino acids (Tyr in NPXXY and Phe in helix 8) to alanine resulted in intracellular retention of the A_1-receptor and a very low receptor surface density. This result is similar to the effect of c-terminal truncation of the receptor protein. As opposed to the wild type receptor, the surface level of the mutant or truncated receptor could not be regulated by overexpression of the receptor chaperone, DRiP78. Nevertheless, the mutant receptors were functional. They were even more susceptible than the wild-type receptor (by at least 5-fold) to activation by a receptor agonist. Similarly, upon deletion of the peripheral portion of the receptor c-tail the truncated receptor revealed enhanced signalling. Modelling suggested that the peripheral segment of the c-tail, replete with acidic side-chains, forms electrostatic bonds with the cytoplasmic face of helix 8 impeding activity-dependent conformational changes.

A20

The Effect of ARNO/Cytohesin-2 on Adenosine A_{2A}-Receptor Signaling in PC-12 Cells

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Recently we found a direct interaction of the guanosine exchange factor ARNO/cytohesin-2 with the carboxyl terminus of the adenosine A_{2A}-receptor. In the present study we asked whether the interaction with ARNO/cytohesin-2 can alter G protein-mediated signaling in pheochromocytoma (PC12) cells. They are a model system for neuronal cells and express A_{2A}-receptors endogenously. Native PC-12 cells were stably transfected with a plasmid coding for a transcription activator protein only active in the presence of tetracycline. Then the cells were stably transfected with a plasmid coding for ARNO (or its dominant negative version) under the control of the tetracycline inducible promoter. We carried out binding studies to identify clones with comparable receptor expression. Overexpression of ARNO or of its dominant negative version did neither change the potency nor the efficacy of the selective A_{2A}-agonist CGS 21680 to stimulate cAMP-accumulation. We monitored the influence of ARNO on the time-course of desensitization and recovery by determining the agonist-dependent cAMP accumulation at various time points after treatment with saturating concentrations of an agonist and agonist withdrawal, respectively. The wild-type form of ARNO delayed the time-course for desensitization, whereas the dominant negative mutant accelerated the recovery of the receptor. We conclude that ARNO/cytohesin-2 plays an important role in the cycle of de- and resensitization of the adenosine A_{2A}-receptor.

A21

Direct Binding of ARNO/Cytohesin-2 to the A_{2A}-Adenosine Receptor Is Necessary for Sustained Activation of the ERK/MAP-Kinase Pathway

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The A_{2A}-adenosine receptor is a prototypical G_s-coupled receptor, but it also signals – e.g. to MAP kinase (mitogen-activated protein) – via a pathway that is independent of heterotrimeric G proteins. Truncation of the carboxyl terminus affects the strength of the signal through these alternative pathways. In a yeast two-hybrid interaction hunt, we screened a human brain library for proteins that bound to the juxtamembrane portion of the carboxyl terminus of the A_{2A}-receptor. This approach identified ARNO/cytohesin-2 – a nucleotide exchange factor for the small (monomeric) G proteins of the ADP-ribosylation factor (ARF) family – as a potential interaction partner. We confirmed a direct interaction by mutual pull-down (of fusion proteins expressed in bacteria) and by immunoprecipitation of the proteins expressed in mammalian cells. In order to circumvent the long term toxicity associated with overexpression of ARNO, we created stable cell lines that stably expressed the A_{2A}-receptor and where ARNO or the dominant negative version E156K-ARNO was inducible by mifepristone. Cyclic AMP accumulation induced by an A_{2A}-specific agonist was neither altered by ARNO nor by the dominant negative version E156K-ARNO. This was also true for agonist-induced desensitization. In contrast, expression of dominant negative E156K-ARNO and of dominant negative T27N-ARF 6 abrogated the sustained phase of MAP kinase stimulation induced by the A_{2A}-receptor. We therefore conclude that ARNO is required to support the alternative, heterotrimeric G protein-independent, signaling pathway of A_{2A}-receptor, that is stimulation of MAP kinase.

A22

Vasodilating Effect of Crude Methanolic Cinnamon Extract on Isolated Bovine Coronary Arteries

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A clinical study revealed Cinnamon as possible dietary supplement for people with type 2 diabetes. Our experiments are based on the fact that diabetes mellitus is a significant risk factor for the development of coronary artery disease (CAD). On bovine coronary arteries (BCAs) we could demonstrate recently that a crude methanolic cinnamon extract (MCE, *Cinnamomum zeylanicum* Nees)

antagonized the contractile responses to several endogenous vasoconstrictors independently on NO and vasodilator prostanoids. The aim of the present study was to further investigate the underlying mechanisms of the vasodilating effect. The relaxation response to MCE (200 µg/ml) was markedly reduced in BCAs pre-contracted with KCl 80 m*M* (11.9 ± 5%) compared with KCl 30 mM (56.3 ± 4.1%). MCE-induced relaxation of U46619 pre-contracted endothelium-denuded BCAs was significantly attenuated by pre-treatment with barium chloride (1 m*M*), an inhibitor of inward rectifier K^+-channels (K_{IR}), and ouabain (0.5 µ*M*), a Na^+/K^+-ATPase inhibitor, whereas other types of K^+-channel blockers had no effect. Furthermore the vasorelaxing activity of MCE was not altered in the presence of the L-type Ca^{2+} channel antagonists nifedipine and diltiazem but almost eliminated when BCAs were pre-contracted with the protein kinase C (PKC) activator phorbol 12,13-dibutyrate (10 µ*M*). It can be concluded, that the vasodilator mechanisms of MCE in endothelium-denuded BCAs appear a) to be partly mediated by hyperpolarization through stimulation of the Na^+/K^+-ATPase and opening of barium-sensitive potassium channels – probably K_{IR} channels – in vascular smooth muscle cells, b) to be inhibited by PKC activation and c) to be not dependent on L-type Ca^{2+} channels.

A23

The Antiepileptic M-Channel Opener Retigabine Is a Noncompetitive Antagonist of Nicotinic Acetylcholine Receptors

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Retigabine suppresses epileptiform discharges in various in vitro and animal models. Its antiepileptic activity is attributed to the opening of M-type potassium channels, but may also involve a potentiation of GABA responses. Here, we show that retigabine inhibits nicotinic acetylcholine receptors (nAChRs). In sympathetic neurons, retigabine reduced [^{3}H]noradrenaline release evoked by the nAChR agonist DMPP, but not that evoked by electrical fields. In whole-cell recordings, retigabine reduced currents evoked by DMPP with half maximal effects at 28 ± 19 µ*M*. The concentration-response curve for DMPP currents was depressed without changes in half maximal activation. The mode of inhibition by retigabine was clearly distinct from that of the open channel blocker hexamethonium. Heterologously expressed α4/β2 nAChRs, the major central type, were blocked by retigabine with similar potency, but skeletal muscle nAChRs were less sensitive, which argues for a subtype selectivity. Gain-of-function mutations in α4/β2 nAChRs are responsible for autosomal dominant nocturnal frontal lobe epilepsy. Hence, retigabine may prove useful in this type of epilepsy.

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A24

Mammalian GTRAP3-18 and Its Role in the Early Secretory Pathway

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GTRAP3-18 has been firstly described as an interacting protein of the neural glutamate-transporter EAAC1. By use of fluorescence microscopy, we determined it as a endoplasmic reticulum (ER) resident membrane-protein. In contrast to the literature, the last amino-acids (KARE) of GTRAP3-18 do not mimic an ER-retrieval motif. This is supported by the fact that GTRAP3-18 failed to bind to Sec24, the cargo receptor protein of the COPII coat that is required for cargo incorporation into COPII-vesicles. GTRAP3-18 slows down ER-to-Golgi transport as determined in a transport assay with the temperature-sensitive viral stomatitis virus glycoprotein. We employed a concentration assay based on the notion that GTRAP3-18 might block cargo concentration in the ER to find the mechanism by which GTRAP3-18 exerts its effect. Indeed, concentration of EAAC1 into transport complexes was reduced by 50% in GTRAP3-18 expressing cells. GTRAP3-18 is strongly related to the prenylated Rab acceptor proteins that regulate intracellular trafficking. It has been shown that Rab1 recruits COPI components to the ER and drives cargo-concentration into vesicular tubular clusters. We reasoned that GTRAP3-18 might block the effect of Rab1. When challenging semi-intact cells expressing EAAC1 alone or together with GTRAP3-18 and Rab1-containing cytosol, we observed that the blocking effect was overcome. In conclusion, we hypothesize that GTRAP3-18 slows ER-to-Golgi transport by blocking the Rab1 activity at the ER.

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A25

Mutants of the Serotonin Transporter Reveal Amino Acids Important for Outward Transport upon Amphetamine Application

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In the present study, we examined the releasing effect of amphetamine (AMPH) and serotonin (5-HT) on distinct mutations of the human serotonin transporter (SERT; T178A, F264C, and T178A-F264C). These mutations have been shown to specifically accelerate inward transport of serotonin [Kristensen et al., 2004]. According to the long-standing paradigm for carrier-mediated efflux, the "facilitated exchange diffusion hypothesis" (Fischer and Cho, J. Pharmacol. Exp. Ther. 1979] the faster a substrate is transported inside, the faster a cytoplasmic substrate should be transported to the extracellular side. HEK 293 cells or *Xenopus laevis* oocytes expressing the SERT wt or the mutant forms of SERT were subject to uptake and efflux experiments as well as to clamp experiments, using the two electrode voltage clamp (TEVC) method. In contrast to the expected acceleration of outward transport elicited by transporter substrates (e.g. 5-HT and AMPH) in SERT mutants, a decrease in outward transport velocity ensued. It is known that efficacy of a substrate for transporter-mediated efflux correlates well with the substrate-induced currents, and also depends on an intracellular rise in sodium. Thus, we applied the TEVC method to measure the sodium-carried current generated by 5-HT and AMPH. In contrast to our expectations, we found the currents elicited by 5-HT rather enhanced than diminished in the mutants – thus, positively correlating with the faster uptake transport velocity. Like 5-HT, AMPH elicited higher currents in the single point mutations compared to wildtype SERT. Interestingly, AMPH-induced currents were almost not detectable in the double mutant. In conclusion, we show that these mutants distinctly interfere with the inward and outward transport mode of SERT.

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A26

The Effect of ARNO/Cytohesin-2 on Adenosine A_{2A}-Receptor Recycling

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The A_{2A}-adenosine receptor activates adenylyl cyclase via $G\alpha_s$ to raise intracellular cAMP levels. In addition, the receptor can signal to the MAP-kinase via a pathway that is independent of the heterotrimeric G protein. Truncation of the intracellular carboxyl terminus of the receptor affects the strength of the signal through these alternative pathways. In a yeast two-hybrid interaction hunt we screened a human brain library for proteins that bound to the carboxyl terminus of the A_{2A}-receptor. This approach identified ARNO/cytohesin-2 – a nucleotide exchange factor for the small (monomeric) G proteins of the ADP-ribosylation factor family – as an interaction partner. We proved a direct interaction by mutual pull-down of fusion proteins expressed in bacteria. Pull-down assays with truncated versions of the carboxyl terminus revealed that the first 22 amino acids are sufficient for the interaction. Likewise, only the C-terminal part of ARNO/cytohesin-2, a pleckstrin homology domain, was necessary to interact with the receptor fusion protein. These findings were confirmed by co-immunoprecipitation of the proteins expressed in mammalian cells. Earlier studies showed that ARNO/cytohesin-2 is important for the internalization of two G_s-coupled receptors, the β-adrenergic and the luteinizing hormone receptor. Based on this notion we conducted biotinylation assays and "antibody feeding" experiments to ascertain the role of ARNO/cytohesin-2 for A_{2A}-adenosine receptor recycling.

A27

L-Type Ca^{2+} Channels (LTCCs) Contributing to High Affinity DHP Binding in Mouse Brain

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In whole brain of $Ca_V1.2DHP^-$ mice in which high DHP sensitivity was removed from $Ca_V1.2$ LTCCs, >80% of the high affinity binding for the LTCC blocker (+)-[^{3}H]isradipine (ISR) are absent and must represent $Ca_V1.2$. We also generated $Ca_V1.2DHP^-$ mice lacking $Ca_V1.3$ channels (double mutants, DM). $Ca_V1.2DHP^-$ mice exhibited 12.6% and DM mice 3.7% of WT ISR binding suggesting that 9% of the binding occurs to $Ca_V1.3$. Using quantitative real-time PCR we could exclude relevant expression of $Ca_V1.1$ and $Ca_V1.4$ (<0.1%) LTCCs. Thus residual binding in DM most likely reflects low affinity binding to genetically modified $Ca_V1.2$. As most of the ISR binding in $Ca_V1.2DHP^-$ mice occurred to $Ca_V1.3$, we determined whether DHPs show selectivity for $Ca_V1.2$ vs. $Ca_V1.3$ by measuring IC_{50} values for ISR binding inhibition in WT and $Ca_V1.2DHP^-$. A 3–5-fold selectivity for the $Ca_V1.2$ component was observed for nitrendipine and nifedipine but not isradipine. This was directly confirmed in binding studies with $Ca_V1.2$ and $Ca_V1.3$ channels expressed in tsA-201 cells. This suggests that the development of isoform-selective modulators should thus be feasible.

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K1

Combined Positron Emission Tomography and Microdialysis for In Vivo Assessment of Intracellular Drug Pharmacokinetics in Humans

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Introduction: We combined positron emission tomography (PET), which measures total concentrations of radiolabelled drugs in tissue, and microdialysis (MD), which determines unbound drug concentrations in the extracellular space fluid of tissue, to describe intracellular pharmacokinetics (PK) of the model compound [^{18}F]ciprofloxacin in vivo. **Methods**: Ciprofloxacin PK were assessed in skeletal muscle of 10 healthy volunteers by means of PET/MD for 5 h after i.v. administration of a mixture of [^{18}F]ciprofloxacin and unlabelled ciprofloxacin (200 mg). A three-compartment PK model was fitted to the ciprofloxacin tissue concentration-time profiles. **Results:** Mean (± SD) total and extracellular C_{max} values of 1.8 ± 0.4 µg/ml and 0.7 ± 0.2 µg/ml were attained at 95 ± 34 and 48 ± 20 min after drug administration, respectively. The extracellular-to-intracellular exchange appeared to be fast with an estimated rate constant k_3 of 1.69 ± 0.25 min^{-1}. An intracellular-to-extracellular concentration ratio of 3.2 ± 0.8 was reached at 110 min p.i. and followed by sustained intracellular retention of the antibiotic. The model-predicted extracellular profiles were in good agreement with the MD data. **Conclusion:** Our results were in accordance with previous in vitro data describing intracellular ciprofloxacin accumulation. The presently employed PET/MD combination might be useful during research and development of drugs, for which knowledge of intracellular concentrations is of interest.

K2

Plasma Concentrations Might Lead to Overestimation of Target Site Activity of Antibiotics

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Introduction: Pharmacokinetic (PK)–pharmakodynamic (PD) models have become increasingly important in optimizing antimicrobial therapy. This approach is highly recommended by regulatory authorities intending to force the evaluation of antimicrobial action at the site of infection. Therefore, in the present study piperacillin, a widely used β-lactam antibiotic, was investigated by a dynamic in-vivo-PK / in-vitro-PD model. **Methods:** Differently susceptible clinical isolates of *Pseudomonas aeruginosa* and *Staphylococcus aureus* with MICs (minimal inhibitory concentrations) of 4, 8 and 16 mg/liter for piperacillin, were employed in the model. Bacteria were exposed in vitro to the concentration-versus-time profiles of piperacillin in plasma and subcutaneous adipose tissue measured in vivo in septic patients. **Results:** Piperacillin levels determined in plasma were able to effectively inhibit bacterial growth of all bacterial strains used in the present study (MIC ranged from 4–16 mg/l). In contrast, concentration-versus-time profiles of subcutaneous adipose tissue were effective to kill isolates with MICs of 4 and 8 mg/l only, while bacterial growth of *S. aureus* and *P. aeruginosa* with MICs of 16 mg/liter was not inhibited. **Conclusion:** The prediction of microbiological outcome based on concentrations of piperacillin in plasma resulted in an overestimation of antimicrobial activity at the site of infection.

K3

Antibiotic Abscess Penetration: Fosfomycin Levels Measured in Pus and Simulated Concentration-Time Profiles

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Introduction: The present study was performed to evaluate the ability of fosfomycin, a broad spectrum antibiotic, to penetrate into abscess fluid. **Methods:** Twelve patients scheduled for surgical or computer-tomography-guided abscess drainage received a single intravenous dose of 8 g of fosfomycin. The fosfomycin concentrations were determined in plasma over time and in pus upon drainage. A pharmacokinetic model was developed to estimate the concentration-time profile of fosfomycin in pus. **Results:** Individual fosfomycin concentrations in abscess fluid at drainage varied substantially, ranging from below the limit of detection up to 168 mg/l. The fosfomycin concentrations in pus of the study population did neither correlate with plasma levels nor with the individual ratios of abscess surface area to volume. This finding was attributed to highly variable abscess permeability. The average concentration in pus was calculated to be 182 ± 64 mg/l at steady state, exceeding the $MIC_{50/90}$s of several bacterial species which are commonly involved in abscess formation like streptococci, staphylococci or *Escherichia coli*. Hereby, the exceptionally long mean half-life of fosfomycin of 32 ± 39 h in abscess fluid may favor its antimicrobial effect, because fosfomycin exerts time-dependent killing. **Conclusion:** After an initial loading dose of 10–12 g, fosfomycin should be administered at doses of 8 g three times per day in order to reach sufficient concentrations in

abscess fluid and plasma. Applying this dosing regimen, fosfomycin levels in abscess fluid are expected to be effective after multiple doses in most patients.

K4

Vitamin C Prevents Endothelial Dysfunction Caused by Ischemia-Reperfusion

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Objective: Ischemia-reperfusion (IR) injury causes tissue injury and endothelial dysfunction. There is evidence that oxidative stress plays an important role. We tested if IR-induced endothelial dysfunction could be prevented by administration of the antioxidant vitamin C. **Methods and Results:** 20 healthy male subjects and 6 male patients with peripheral arterial disease (PAD) were enrolled in this randomised placebo-controlled study. Forearm blood flow (FBF) measurements in response to the vasodilators acetylcholine (ACh; endothelium-dependent agonist) or nitroglycerin (NTG; endothelium-independent) were performed before and after forearm ischemia for 20 minutes. FBF responses were reassessed during reperfusion with intra-arterial co-administration of 24 mg/min vitamin C or placebo. ACh-induced vasodilation was blunted in subjects receiving placebo after reperfusion ($p<0.05$ vs. baseline). Administration of vitamin C completely prevented impaired responsiveness. NTG-induced vasodilation was not affected by reperfusion or vitamin C. This finding was consistent in patients with PAD and impaired endothelial function, where local vitamin C infusion restored FBF reactivity to ACh before and after IR injury ($p<0.05$ vs. baseline). Again, NTG-induced vasodilation was not affected. **Conclusions:** Our data indicate that IR-induced vascular injury can be prevented by administration of antioxidants.

K5

Recombinant Human Antithrombin Has Anticoagulant and Anti-Inflammatory Properties in Human Endotoxemia

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Introduction: We hypothesized that infusion of recombinant human antithrombin without concomitant heparin would have dose-dependent anticoagulant properties and potentially decrease endotoxin (LPS)-induced cytokine production. **Methods/Results:** The study was randomized, double-blind, placebo-controlled in parallel groups enroling 30 healthy male volunteers. The active treatment groups received infusions of recombinant human antithrombin (rhAT) to increase antithrombin (AT) levels to 200% and 500% before infusion of 2 ng/kg LPS, which dose-dependently decreased coagulation ($p<0.01$, ANOVA): peak levels of prothrombin fragment (F_{1+2}) (1.8 nmol/L in the 500% AT group and 4.4 nmol/L in the placebo group at 4h), thrombin antithrombin complexes (TAT) (12 µg/L and 34 µg/L at 4 h, respectively) and D-dimer (0.2 µg/L and 0.5 µg/L, respectively). RhAT decreased peak interleukin-6 (IL-6) levels by 40% (222 pg/mL and 216 pg/mL in the 500% and 200% AT group, respectively, versus 357 pg/mL in the placebo group, $p<0.01$, ANOVA). Finally, infusion of rhAT rapidly decreased neutrophil (by 19% in the 500% AT group versus 6% in the placebo group; Kruskal Wallis ANOVA $p=0.002$) and monocyte counts (by 30% in the 500% AT group and by 18% in the 200% AT group versus 8% in the placebo group; $p=0.04$) before LPS-challenge. **Conclusion:** In summary, rhAT dose-dependently inhibited tissue factor-triggered coagulation. Effects on leukocytes and IL-6 release seem to represent specific properties of rhAT.

K6

Effects of Carbon Monoxide Inhalation during Experimental Endotoxemia in Humans

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Recent data show that carbon monoxide (CO) exerts direct anti-inflammatory effects in vitro and in vivo after lipopolysaccharide (LPS) challenge in a mouse model. We hypothesized that CO may act as an anti-inflammatory agent in human endotoxemia. The aim of this trial was to study the effects of CO inhalation on cytokine production during experimental human endotoxemia. The main study was randomized, double-blinded, placebo-controlled two-way crossover in healthy volunteers. Each volunteer inhaled synthetic air (as placebo) and 500 ppm CO for 1 hour in random order with a washout period of 6 weeks and received a 2 ng/kg intravenous bolus of LPS after inhalation. HbCO levels were assessed as a safety parameter. CO inhalation increased HbCO levels from 1.2% [95% CI: 1.0–1.4%] to peak values of 7.0% [CI: 6.5–7.7%]. LPS infusion transiently increased plasma concentrations of TNF-α, IL-6 (~150 fold increases), IL-8 as well as IL-1α+β mRNA levels (~200 fold increase). These LPS-induced changes were not influenced by CO inhalation. Inhalation of 500 ppm CO for 1 hour had no anti-inflammatory effects in a systemic inflammation model in humans as 250 ppm for 1 hour did in rodents.

K7

A Bioequivalence Study of Two Oral Desmopressin Tablet Formulations

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Objective: The present study was carried out to test the bioequivalence between two different oral desmopressin formulations. As the bioavailability of peptides such as desmopressin markedly varies within and between subjects, reasoned sample size estimation in combination with a sensitive analytical method for the measurement of plasma concentrations are prerequisites for a conventional pharmacokinetic bioequivalence study. **Methods:** 30 male and 30 female healthy volunteers (n = 60) were enrolled in the study and were randomly assigned to receive the test- and reference drug on two occasions in a crossover study design. Subjects received an oral single dose of 400 µg (2 tablets of 200 µg) of desmopressin-acetate per study day separated by a wash-out period of at least seven days. Desmopressin plasma concentrations were measured in intervals over a 12-h period using a validated radioimmunoassay method. The main pharmacokinetic parameters, the AUC and the C_{max} were analyzed by ANOVA after log-transformation. The 90% confidence intervals of the AUC and C_{max} test/reference ratios were stipulated to lie in the 0.80–1.25 interval. **Results:** The mean test/reference drug ratios were within the bioequivalence boundaries with mean values of 0.99 [90% CI: 0.87–1.14] and 1.03 [0.92–1.15] for AUC_{0-t} and AUC_{0-inf}, respectively. For the C_{max}, the mean ratio of test/reference drug was 0.97 [0.87–1.08]. The safety and tolerability of both formulations were equivalent. **Conclusion:** The rate and the extent of absorption are identical for both desmopressin formulations. Thus, the desmopressin test tablet met all equivalence criteria and thereby was proven bioequivalent with the marketed reference desmopressin tablet.

K8

CCI-779 plus Cisplatin Is Highly Effective against Human Melanoma in a SCID-Mouse Xenotransplantation Model

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This study explored the anti-neoplastic effect of CCI-779 administered as mono-therapy and in combination with the standard chemotherapeutic agent cisplatin in a human melanoma SCID-mouse xenotransplantation model. 24 mice per tumor cell line were used in a controlled four groups (6 mice per group) parallel study design. Thus, a total of 72 SCID mice were injected either with 5 x 10^6 518A2, Mel-JUSO or 607B human melanoma cells in the lower flank. After development of palpable tumors, mice received daily intraperitoneal injections with CCI-779 (1.5 mg/kg) or solvent over a period of 14 days. On days 2 and 6, they were additionally injected with cisplatin (5 mg/kg i.p.) or saline, which served as control. After two weeks, the combined treatment with CCI-779 plus cisplatin eradicated completely 4 of 6 established 518A2 melanomas grown in SCID mice. The combined administration of CCI-779 plus cisplatin synergistically reduced the tumor weight in the remaining 518A2 xenografts by ~90% ($p<0.05$) when compared solvent control. A significant anti-tumor effect was also detected in vivo for the Mel-JUSO and 607B melanoma cell lines. CCI-779 mono-therapy resulted in a tumor weight reduction of approximately 20–30% in 518A2, Mel-JUSO and 607B xenografts ($p<0.05$). Notably, treatment with cisplatin only exerted no significant anti-neoplastic effect. TUNEL analysis showed an approximately 5-fold increase of apoptotic cells in xenografts treated with CCI-779 plus cisplatin compared to solvent control. We conclude that the combined treatment with CCI-779 plus cisplatin eradicates or effectively inhibits human melanoma grown in SCID mice.

K9

Intravenous Administration of L-Arginine Increases Retinal and Choroidal Blood Flow

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Purpose: Nitric oxide (NO) is among the most important regulators of ocular perfusion. L-Arginine, an amino-acid, is the precursor of NO synthesis. The aim of the present study was to determine whether administration of L-Arginine affects ocular blood flow. **Design:** L-arginine (1 g/min) or placebo was administered intravenously for 30 minutes in 12 healthy volunteers in a randomized, double masked, two way cross over design. **Methods:** Ocular haemodynamics were measured before, in the last 10 minutes of the infusion period as well as 30 minutes after cessation of the administration. Retinal vessel diameters were measured with a Retinal Vessel Analyzer, red blood cell velocities with bi-directional laser Doppler velocimetry and pulsatile choroidal blood flow was measured using laser interferometry. **Results:** L-arginine significantly decreased mean arterial pressure by −8 ± 5% and −6 ± 7% at the two time points ($p<0.01$), respectively. Intravenous administration of L-arginine increased choroidal blood flow by +10 ± 6% and +12 ± 7%. Retinal venous diameters decrease by −2.5 ± 2.1% and −1.4 ± 2.7% whereas red

blood cell velocity, significantly increased after administration of L-arginine by +22 ± 23% and +20 ± 19%. Thus, calculated blood flow in retinal veins, increased by +21 ± 18% and +21 ± 19% before and after the end of L-arginine infusion. **Conclusion:** Intravenous administration of L-arginine increases retinal and choroidal blood flow in healthy volunteers. Whether this effect is related to an increased NO production or an unidentified mechanism remains to be clarified. However, administration of L-arginine might be an interesting new approach to therapeutically increase ocular blood flow in ocular vascular disease.

M1

Modelling Drug Effect on Sodium Channels

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We studied the inhibitory effect of various drugs on voltage-gated sodium channels on cultured rat hippocampal neurons using whole cell voltage clamp technique. Based on the hypotheses regarding the mechanism of action of different drugs we created a model of the sodium channel, which, besides being able to simulate the voltage-dependent gating behavior of sodium channels, is also able to incorporate drug effects of different mechanisms, and concentrations. We illustrate in this study the effects of the antidepressant fluoxetine, and the anticonvulsant carbamazepine. Both drugs inhibit the sodium channels in a use-dependent, and voltage-dependent manner, but with slight differences. We hypothesized that the differences in the properties of inhibition by the two drugs are due to a different affinity profile. The carbamazepine preferentially binds to the fast, the fluoxetine to the slow inactivated state. In our model, we incorporated two voltage-independent and six voltage-dependent mechanisms. We assumed that drug binding is possible to all conformational states, and drug binding alters specific gating rate constants. We performed simulations with fast and slow inactivated state-preferring drugs, and reproduced the small but characteristic differences between the effects of fluoxetine and carbamazepine that were observed experimentally.

M2

The Role of Vagal Nerve in the Insulin Sensitivity

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The effect of vagus nerve function on the post-prandial activation of the hepatic insulin sensitizing substance (HISS) mechanism was investigated in healthy male Wistar rats using the rapid insulin sensitivity test or the hyperinsulinaemic euglycaemic glucose clamp (HEGC) method. In fed animals, electrical stimulation (square impulses: 25 V, 5 Hz, 0.5 ms over 15 min) of the vagus nerve induced hyperglycaemia and an increase in plasma insulin immunoreactivity. Atropine (1.0 mg/kg intravenously) induced insulin resistance estimated by rapid insulin sensitivity testing. This was amplified when the vagus nerve was stimulated. The insulin resistant state developed as a result of fasting and it was not modified by either treatment with atropine or electrical stimulation. The role of vagal sensory afferents was investigated by means of capsaicin pretreatment of the cervical part of the left vagal nerve. After capsaicin pretreatment, the metabolic variables (body weight, consumed food and tap water, daily diuresis and defecation) were measured over 14 days by means of metabolic cages. At the end of the study period, the insulin sensitivity was determined by HEGC. Capsaicin pretreatment evoked significant changes in daily food and water consumption as well as urine production while the body weight and the stool production was left unaffected and the insulin sensitivity was worsened as compared with the solvent-treated group and the latter was 14.4 ± 4.3 and 20.4 ± 6.9 mg/kg/min, respectively. We conclude that both parasympathetic cholinergic and non-cholinergic vagal efferents modulate post-prandial neurogenic insulin sensitivity adjustments. Insulin sensitivity and metabolic variables of the rat is modulated at least in part by capsaicin-sensitive sensory vagal afferents.

M3

Gastrointestinal (GI) Safety of Low Dose Diclofenac-Potassium Compared to Low Dose Ibuprofen

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The GI profile of low-dose diclofenac was assessed from clinical trials, endoscopic studies and epidemiological data. Data from 15 randomised multiple-dose clinical trials, 5 with low-dose diclofenac-K (≤75 mg/day) (n = 1297), 8 with high-dose diclofenac-K (>75 mg) (n = 1174) in acute indications and 2 with low and high doses in chronic indications, controlled with ibuprofen (≤1200 mg, n = 873; >1200 mg, n = 247), or placebo (n = 1022), showed a clear relationship between dose, treatment duration and indication. With short-term low dose diclofenac-K, ibuprofen the incidence rates of total GI events in acute indications were 6.7 (4.8–9.1) and 6.5 % (4.5–9.1), respectively. There was also no difference with short-term high dose in chronic conditions or long-term high dose in chronic conditions. In endoscopic studies (n = 12, healthy volunteers, 5 days), mean Lanza gastric scores in the low-dose diclofenac-K (0.33 ± 0.5) and low-dose ibuprofen (0.42 ± 0.7) groups were similar and significantly better than in the ASA 3000 mg/day group (2.67 ± 0.9). Differences reported in five large pharmacoepidemiological

studies of NSAID-related GI events looking at dosage (low-dose ibuprofen safer than diclofenac) could be related to different indications since in these studies ibuprofen was available OTC and diclofenac only on prescription. Overall for the same indication, duration and dose there was no evidence of difference in the incidence or relative risk of GI events between low-dose diclofenac and ibuprofen.

M4

Inhibition of Neuronal Sodium Channels by the Antidepressants Fluoxetine and Desipramine Involves Stabilization of Slow Inactivated State

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The effect of the antidepressants fluoxetine and desipramine on sodium channels of rat hippocampal neurons was investigated. The drugs were roughly equipotent use-dependent inhibitors of sodium channels. IC_{50} values were 107.9 and 83.4 μ*M* at –150 mV, while 1.11 and 1.68 μ*M* at –60 mV, for fluoxetine and desipramine, respectively. The inhibition was not only dependent on the actual membrane potential, but also on previous temporal pattern of it. To be able to assess the potency during a certain in vivo activity pattern, a knowledge of the mechanism of action is essential. We recently described a novel mechanism of use-dependent sodium channel inhibition, in which the slow but not the fast inactivated state of sodium channels is stabilised by the drug, and significant association to resting channels is possible. We found that these antidepressants inhibit sodium channels via this novel mechanism, because (i) association of fluoxetine and desipramine affected neither the rate of fast inactivation nor the recovery from it; but it markedly affected slow inactivation and the rate of recovery from slow inactivated state; and (ii) association to slow inactivated state caused larger inhibition than association to fast inactivated state. We suggest that therapeutic concentrations of antidepressants affect neuronal information processing partly by a direct, activity-dependent inhibition of sodium channels.

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M5

Effect of Fluoxetine on Hippocampal Delayed Rectifier and A-Type Potassium Channels

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The effect of fluoxetine was investigated on potassium channels of rat hippocampal neurons using the whole-cell patch-clamp technique. We have demonstrated previously that fluoxetine (and other antidepressants) inhibit sodium channels at therapeutical concentrations in a highly activity-dependent manner. This suggests that beside its well-known serotonin transporter inhibitor action, direct effects of fluoxetine on different types of ion channels might play a role in its therapeutic effect. In an effort to understand the complex modulation of neuronal function in the presence of low micromolar concentration of fluoxetine, we studied how native potassium channels of hippocampal neurons are affected by fluoxetine. We separated three types of potassium currents by specific voltage protocols as described by Ficker and Heinemann [J. Physiol. 1992; 445: 431–455]: Fast and slow transient as well as delayed rectifier-type potassium currents. Fast transient currents were minimally affected by up to 30 μ*M* fluoxetine. Slow transient currents were inhibited by both a decreased amplitude and an accelerated decay. The amplitude of delayed rectifier-type currents was similarly decreased. Our results suggest that in order to understand how neuronal information processing is altered by the acute effects of fluoxetine, we need to integrate consequences of activity-dependent modulation of different ion channels.

Support: Hungarian Research Fund (OTKA T037659, T046827)

T1

Enhanced Stability of Oxygen-Centered Radical Spin Adducts from Derivatives of 5-Ethoxy-Carbonyl-3,5-Dimethyl-Pyrroline N-Oxide

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Since radicals are playing a major role in the onset of many diseases, the development of adequate spin traps for their detection is therefore becoming increasingly important. In analogy to frequently used spin traps such as DMPO ($t_{1/2}$ ca. 45 sec) a series of novel compounds has recently been reported to form superoxide spin adducts with significantly higher half-lifes ($t_{1/2}$ ca. 1–25 min) at physiological pH. A series of novel derivatives of the spin trap 5-ethoxycarbonyl-5-methyl pyrroline N-oxide (EMPO) bearing an additional methyl group in position 3 or 4 of the pyrroline ring was synthesized in order to further increase the stability of radical adducts, especially with respect to the detection of oxygen-centered radicals such as the superoxide or alkoxyl radicals, which are frequently formed during the process of lipid peroxidation. In order to provide a whole set of spin traps with increasing lipophilic properties, the ethoxycarbonyl group of EMPO was also replaced by either a propoxy- or an isopropoxy group. The structure of all compounds was confirmed by ^{1}H and ^{13}C-NMR. The stabilities of the superoxide adducts of these novel compounds were significantly higher than those of EMPO or DEPMPO ($t_{1/2}$ ca. 15–40 min). In addition, spin

adducts obtained from radicals derived from methanol, ethanol, formic acid and linoleic acid hydroperoxide were also investigated.

The research was supported by the Austrian "Fonds zur Förderung der wissenschaftlichen Forschung".

T2

Investigation of the Toxicity of Novel EMPO Derivatives towards Different Cell Lines

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Free radicals are playing a major role in different regulatory and pathological processes. Especially detection of superoxide in living cells or whole organisms using ESR spin trapping is of interest. Therefore the design of adequate spin traps with low toxicity and high selectivity is necessary. A series of novel derivatives of the spin trap 5-ethoxycarbonyl-5-methyl pyrroline N-oxide (EMPO) was synthesized. Stabilities of their superoxide adducts were higher than those of EMPO or DEPMPO ($t_{1/2}$ ~15–55 min). Our study investigates the toxicity of this novel compounds to cultured human colon carcinoma cells (SW480), lung carcinoma cells (A549), breast carcinoma cells (SKBR3) and fibroblasts (F2000). A dose-dependent decrease of the cell number was observed for all spin traps. At 50 m*M* 3BEMPO, 4BEMPO and 4,5DPPO caused pronounced cell loss (>90%) and increased LDH-release. 4,5DiPPO caused 47% cell loss and and increased LDH-release as well. The use of 50m*M* 3,5EDPO, 4,5EDPO, 3,5DPPO and 3,5DiPPO caused only moderate cell loss (<25%) without significant effects on LDH-release. Our data indicate necrotic cell death possibly due to membrane toxicity. The following toxicity ranking was obtained: 4,5-DPPO > 3BEMPO ~ 4BEMPO > 4,5DiPPO > 3,5DPPO ~ 3,5EDPO ~ 3,5DiPPO > 4,5EDPO. The least toxic compound was 4,5EDPO with LD_{50}=151 m*M*. iPrMPO and sBuMPO ($t_{1/2}$=19–26 min) were also well tolerated. In conclusion, up to 50 m*M* 4,5EDPO, 3,5DPPO, 3,5EDPO and 3,5DiPPO ($t_{1/2}$=45–55min) can be recommended for further investigation of superoxide radicals in biological systems.

The research was supported by the Austrian "Fonds zur Förderung der wissenschaftlichen Forschung".

T3

Inhibition of the Mitochondrial bc_1 Complex by Oxidation Products of Chromanols

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Lipid peroxidation of mitochondrial membranes is inhibited by the cooperative antioxidative action of the natural chromanol α-tocopherol (Toc) and ubiquinol (UQH_2). The consumption of Toc results in its irreversible oxidation to α-tocopheryl quinone (TQ). In contrast, ubiquinone (UQ) can effectively be recycled to UQH_2 by mitochondria. Therefore, it is likely that most TQ in mammalian cells is formed by the antioxidative activity of Toc. This was supported by our findings in rat liver mitochondria showing that 3.5 ± 0.3% of α-tocopherol was oxidized to TQ in the inner mitochondrial membrane due to high oxidative stress whereas only 0.4 ± 0.05% TQ was present in the outer membrane. In previous studies, it was observed that due to the structural similarity of TQ with UQ TQ interferes with the electron transfer functions of UQ at the isolated bc_1 complex. There exists also a chromanol analogue of UQ; however, its bioactivity is widely unknown. Therefore, we synthesized ubichromanol (UCa) from natural UQ10. The radical scavenging rate constant for this chromanol with the model radical diphenyl picryl hydrazyl was about 50% smaller in comparison with Toc in ethanol. The major oxidation product of UCa arising from the antioxidative reaction was identified by means of HPLC/MS, ^{1}H and ^{13}C-NMR as a new UQ derivative with a hydroxylated side chain (UQ10OH). Experiments with the isolated bc_1 complex revealed that UQ10OH has inhibiting properties on the electron transfer from UQH_2 to cytochrome c as well.

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T4

Proteome Analysis Identifies Kupffer Cells as Direct Target of the Hepatocarcinogen N-Nitrosomorpholin

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The molecular mechanisms of hepatocarcinogenesis are not well understood. Although hepatocytes are the sole cell type found to undergo cell transformation in liver cancer, recent data have described a critical contribution of Kupffer cells (KC) to the effects of chemical carcinogens. Here, we applied a highly sensitive proteome analysis as a powerful

approach to investigate the effect of the well-described hepatocarcinogen N-nitrosomorpholin (NNM) on hepatocytes and KC. We studied very early effects of NNM in vivo on rat liver, as well as on isolated primary hepatocytes and KC. Furthermore, primary cells were treated in vitro with NNM. Cells were metabolically labelled with ^{35}S-methionine/cysteine to study synthesis rates of proteins. Subsequently, cytoplasmic proteins were separated by 2D gel electrophoresis and analysed by fluorescence detection, autoradiography and mass spectrometry. We found that indeed both cell types, hepatocytes and KC, responded to NNM treatment in vivo and in vitro by a pronounced general induction of protein synthesis in addition to more specific protein regulations. The observed NNM-induced cell activations were quantitatively comparable with the effects of inflammatory activators IL-6 and LPS on hepatocytes and KC, respectively. KC activation by NNM appeared to be stronger than that of hepatocytes. Our results demonstrate that KC are a direct target for NNM toxicity and may critically contribute to hepatocarcinogenesis.

T5

The Role of NADPH Oxidase in Kupffer Cell Superoxide and Liver Nitrogen Oxide Production in Response to the Hepatocarcinogen Diethylnitrosamine (DENA)

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Nitrosamines occur in traces in food or may be generated endogenously. They are one chemical factor involved in hepatocarcinogenesis, a process mediated by genotoxic and cytotoxic events. Cytotoxic reactive oxygen species (ROS) and TNF-α production, and DNA damage are increased after (DENA) treatment in wild type (wt) mouse liver but not in p47-NADPH oxidase knockout (phox-ko) mice [Teufelhofer et al., Carcinogenesis 2005; 26: 319–329]. Furthermore, in a recent study we pre-treated wt mice with apocynin, a phox inhibitor. Under these conditions superoxide (SO) production after DENA was drastically decreased. We hypothesized that besides ROS also nitric oxide may be involved in the cytotoxic and inflammatory actions. Therefore, we examined liver proteins for nitrated derivatives. These were found by Western analysis at early time points after DENA in wt, but not in phox-ko mice. In addition, mRNA expression of iNOS was examined by RT-PCR. Interestingly, mRNA levels were not changed in wt but were increased in phox-ko males only. In contrast, in female mouse livers of both strains mRNA levels remained unaltered. New hypothesis: Lacking superoxide generation in male phox-ko is counterbalanced by increased iNOS expression.

T6

Interspecies Comparison of Ras-Inactivation in Hepatocarcinogenesis

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The morphology of hepatocellular carcinoma is largely similar among rodents and humans. The molecular pathogenesis, however, often reveals species-differences complicating the interpretation of results of rodent bioassays for chemical hepatocarcinogenicity; e.g., chemically-induced rodent liver tumors frequently harbour activating point mutations in one of the Ras-oncogenes, while in human hepatocellular carcinoma (HCCs) the frequency of Ras-mutations is low. The putative tumor suppressor genes, NORE1A/B, and RASSF1A, are part of a regulatory mechanism that antagonizes the growth-enhancing effects of the proto-oncogene Ras. In human HCCs we studied the possible inactivation of NORE1A/B and RASSF1A by promoter hypermethylation (through methylation-specific PCR) and by inactivating mutations (through single-strand conformational polymorphism and sequencing). No evidence for mutations could be found for NORE1B, NORE1A and RASSF1A. Considerable CpG-methylation was found for NORE1B and/or RASSF1A in 96% of HCCs, but not for NORE1A. DNA methylation and absent expression of NORE1B and RASSF1A was also evident in many hepatoma-cell lines and could be restored by 5-aza-cytidine (prevents DNA-methylation). Our data suggest that epigenetic silencing of NORE1B and RASSF1A is a frequent event in human hepatocarcinogenesis and may exert effects similar to activating mutations of Ras. Thus, the Ras pathway is affected in hepatocarcinogenesis of both, rodents and humans.

T7

Green Fluorescent Protein-Expressing Adenovirus – a Useful Tool to Study Viral Stability during Laboratory Experiments

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Adenoviruses are widely used vectors in life science research and they are promising tools for human gene therapy. However, other than for pharmaceutical productions, only little is known on adenoviral stability during small scale laboratory experiments. In this study we describe the application of a recombinant, replication-deficient green fluorescent protein (GFP)-expressing adenovirus in combination with fluorescence-activated cell

sorting (FACS) as a simple and effective tool for monitoring viral infectivity under different experimental conditions. Applying this system we could demonstrate that adenoviruses remain infective under common cell culture conditions (37°C) for several days, but heat treatment (5 min, 95°C), desiccation (24 h) or direct UV radiation (30 min) is sufficient to deactivate viral particles. Further we could show that some fixatives, protein extraction buffers, and reagents used for processing of cells are sufficient to eliminate infectious particles, while others hardly affect infectivity. In conclusion the data collected in this study provide valuable information for other laboratories that are working with adenoviral vectors and the described application of GFP-expressing adenoviruses is a convenient way to develop adequate decontamination procedures for experiments with adenoviruses.

T8

The Coffee Diterpenes Kahweol and Cafestol Mitigate Acute Toxicity and Oxidative Effect of the Alkylating Carcinogen Azoxymethane in Rat Liver

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The lower rate of cancers of colon and liver in coffee drinkers may in part be caused by intake of the diterpenes kahweol and cafestol (K/C). K/C were found to cause several beneficial effects in vivo and in vitro, predominantly modifications of carcinogen-activating and detoxifying enzymes. Consequently, chemopreventive effects were observed against several carcinogens of great human relevance. Since K/C was also found to enhance the repair capacity towards alkylated DNA, further research was started to investigate the complete picture of potential protection by K/C against alkylating carcinogens such as azoxymethane (AOM). Male F344 rats were pretreated with 0.2% K/C for 10 days in their feed followed by a carcinogenic i.p. dose of AOM (30 mg/kg body weight) and were compared with positive controls receiving only AOM and with negative vehicle controls. The rats were sacrificed before and at 12, 24, 36, and 48 hrs after AOM injection. Hepatotoxicity, as monitored by serum ALT and liver histology, was encountered in the positive controls which was however clearly mitigated in the animals pretreated with K/C. From 24 h post-injection on, AOM enhanced total lipid hydroperoxides in the liver up to >3-fold (36 and 48 h) and, again, K/C-pretreatment led to weakening of this effect to ca. 2-fold. Thus, oxidative stress has likely contributed to the acute toxicity of AOM at the later time points investigated and K/C has apparently exerted protection partially as an antioxidant. This is in agreement with the earlier observed increased synthesis of hepatic glutathione caused by K/C. The possible involvement of ferritin in this antioxidant mechanism is also investigated.

Author Index *[published abstracts only]*

Numbers refer to Abstract No.

Late abstracts:

L1

Induction of Oxidative Stress by Acrylamide Is Related to the Differentiation Grade of Caco-2 Cells

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Objectives: Acrylamide is metabolized via cytochrome P450 2E1 (CYP2E1) to glycidamide (phase I), a chemically reactive epoxide that forms DNA adducts. This metabolite is detoxified via glutathione-S-transferase (GST) to N-acetyl-S-(3-amino-3-oxopropyl) cysteine (phase II). Caco-2 cells express both, CYP2E1 and GST, but expression of the latter is dependent on the degree of differentiation, for which apical alkaline phosphatase expression is a marker. **Methods:** In the presented study Caco-2 cells were grown until different stages of confluency (day 3, 7) and differentiation (day 3, 7, 14, 21). Cells were incubated for 24 hours with 0.1, 0.5 and 1 m*M* of acrylamide dissolved in deionized water added directly into the culture medium. Thereafter activity of GST and alkaline phosphatase as well as reduced/ oxidized glutathione GSH/GSSG ratio and lipid peroxidation were analyzed. **Results and Discussion:** GST and alkaline phosphatase activities increased during differentiation of the cells while addition of acrylamide showed no effects. Glutathione levels decreased until confluency was reached (day 7). At day 14 and 21 (day 21: cells are fully differentiated) acrylamide significantly decreased total glutathione levels and at day 14, also the GSH/GSSG ratio decreased in a concentration dependent manner. This indicates oxidative stress burden. However, regarding lipid peroxidation all samples analyzed were below the detection limit. **Conclusion:** The present data suggest that, by reducing glutathione levels, acrylamide or its metabolite might affect phase II detoxification in differentiating Caco-2 cells. Additional measurements of CYP2E1 expression are currently performed to assess the role of this CYP in acrylamide induced oxidative stress.

L2

Interference of Tocopheryl Quinone with Mitochondrial Electron Transfer

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Vitamin E functions as a radical-trapping antioxidant in mitochondria which are believed to release superoxide as a byproduct of respiration. Oxidized tocopherol which escapes recycling by ascorbate or ubiquinol is irreversibly converted to tocopheryl quinone (TQ). We analyzed the amounts of TQ in submitochondrial fractions from healthy rats and found ca. 10 pmol TQ per mg protein in mitoplasts and outer mitochondrial membranes, which translates into a ca. 10-fold smaller TQ:lipid ratio in the latter. This underscores the protective role of tocopherol at the site of cell respiration. Since TQ resembles ubiquinone (UQ), we studied the extent to which short-chain TQ_1 competes with UQ_1 in mitochondrial electron transport, using submitochondrial particles from beef heart. Complex I (NADH dehydrogenase) turned out to reduce TQ_1 in a rotenone-sensitive manner, although with smaller affinity (K_M 115 μ*M* versus 23 μ*M*) and 10-fold reduced rate (v_{max} 23 versus 260 nmol/mg/min). On the other hand, no reactivity was found for complex II (succinate dehydrogenase) due to its more specific quinone binding pocket. Complex III reduced TQ_1 in the course of the Q cycle. This antimycin A-sensitive reaction was 100 times slower compared to UQ_1, but the affinities of the two substrates were comparable (K_M 36 and 22 μ*M* for TQ_1 and UQ_1, respectively). The results suggest a weak competition of TQ, especially under conditions of oxidative stress, and also provide a mechanism for the generation of reduced TQ which itself is a strong antioxidant.

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L3

Fine Particulates and Cardiopulmonary Incidents in Vienna

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In earlier smog episodes SO_2 proved to be the indicator of air pollution with highest correlation to daily mortality and morbidity [Wien. Klin. Wochenschr. 2004; 116 (Suppl. 1): 8–12]. After its successful reduction focus shifted to particulate pollutants and time series studies found dose-response relationships with fine particle mass without indication for a threshold. In Vienna daily hospital admissions of chronic pulmonary diseases (ICD-9: 490–496) in 1999/2000 increased in persons aged 65 years and older with $PM_{2.5}$ concentrations [Atmos. Environ. 2004; 38: 3971–3981]. The aim of this study was to analyse daily mortality in Vienna 2000–2004 with methods used in studies of other European cities [Am. J. Respir. Crit. Care Med. 2004; 169: 1257–1258]. Polynomially distributed lag models for up to 14 days were used (Poisson GAMs), applying stringent convergence criteria and considering seasonal, daily meteorological influences and gaseous pollutants as confounders. Per 10 μg TSP/m^3 the increase of total daily mortality (lagged 0–14 days) was 1.2% and for elderly aged 65+ it was 1.5%. An increase of 3.7% (3.9% at

age 65+) was found for ischemic heart disease (ICD-9: 410–414) and of 7.6% for chronic pulmonary disease (ICD-9: 490–496). The contributions of particle subfractions still have to be determined. NO_2 proved to be an independent indicator of urban air pollution increasing cardiopulmonary incidents. Reduction of both fine particulates and NO_2 need to be continued; outdoors mainly by control of motor traffic and indoors by tobacco control, cleanup of workplaces and ventilation of gas stoves.

L4

Coffee Protects Peripheral Human Lymphocytes Against Oxidative DNA-Damage

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Aim of the study was the investigation of the potential DNA-protective effects of coffee consumption in humans. DNA damage was monitored in lymphocytes of eight individuals with the single cell gel electrophoresis assay before and after consumption of 600 ml coffee/day over five days. Under standard conditions, no alteration of DNA migration was seen, but a pronounced reduction of DNA migration attributable to endogenous formation of oxidised purines and pyrimidines was detected with restriction enzymes; furthermore induction of DNA damage induced by hydrogen peroxide and by heterocyclic aromatic amines was significantly reduced after coffee consumption. Also in in vitro experiments with lymphocytes, inhibition of H_2O_2 induced DNA damage was found with coffee at low concentrations whereas coffee diterpenoids caused only marginal effects. These findings indicate that the effects in humans are partly due to scavenging effects of constituents other than diterpenoids. Enzyme measurements showed that also induction of antioxidant enzymes may play a role: while the activity of glutathione peroxidase in cytosols of peripheral lymphocytes was only marginally increased at the end of the intervention period, a significant increase of superoxide dismutase activity was detected. Comparisons of our findings with results of earlier studies support the assumption that coffee consumption prevents oxidative DNA damage to a higher extent as fruits and vegetables.

L5

Consumption of Brussels Sprouts Protects Peripheral Human Lymphocytes against 2-Amino-1-Methyl-6-Phenylimidazo[4,5-b]-Pyridine (PhIP): Results of a Controlled Human Intervention Trial

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Heterocyclic amines (HAs) are formed during cooking of meats and might be involved in the aetiology of various forms of human cancer. Due to the low exposure levels, it is not possible to monitor prevention of HA adduct formation in humans. As an alternative we developed a protocol in which we monitored the alterations of the sensitivity of peripheral human lymphocytes in single cell gel electrophoresis assays with PhIP (the most abundant HA in fried meat) and Trp-P-2 (which is less abundant but a stronger carcinogen in rodents). We used this model to study the effects of Brussels sprout consumption on HA-induced DNA damage. Eight volunteers consumed 300g/d of steamed sprouts over five days. Lymphocytes were isolated at the beginning and at the end of the study and were exposed to the amines for 30 min. PhIP-induced DNA migration was inhibited significantly after consumption of the vegetables, whereas the sensitivity of the cells towards Trp-P-2 was not affected. Since genetically engineered cells showed that PhIP but not Trp-P-2 requires activation via sulfotransferases, we monitored the effect of Brussels sprout consumption on SULT (1A1, 1A3) in Western blots and enzyme assays in a further trial and found strong inhibition of these enzymes. Our findings provide an explanation for the prevention of PhIP DNA adduct formation seen by sprouts in animals and suggest that the consumption protects humans against PhIP induced DNA damage via inhibition of sulfotransferases.

L6

In Silico Toxicology: Current Status and Future Perspectives

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The term „in silico toxicology" generally stands for computer assisted toxicological and ADME assessment of chemical substances as well as the development of corresponding computer models and software tools. Two general approaches can be identified: on the one hand fine tuned expert systems exist that try to qualitatively guess substance effects on classical toxicological endpoints, on the other hand mechanistic models are increasingly

developed that focus on quantitative prediction of individual aspects in the overall toxicological (or ADME) picture. While it would of course be highly desirable to apply mechanistically well founded quantitative models also for "global" toxicity prediction this is currently hampered by the extraordinary complexity of underlying phenomena. However, toxicogenomics, -proteomics and systems biology approaches increasingly contribute data on which refined mechanistic models can be built that also incorporate structural aspects of involved enzymes, receptors and other proteins. It can be estimated that in the future in silico toxicology will not only continue to gain importance in industrial drug development, but will also reach regulatory acceptance as a tool to assist in data evaluation as well as for prioritisation of experimental testing.

L7

Genotoxic Effects of Common Mycotoxins Cause DNA Damage in Human Derived Hepatoma (HepG2) Cells

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It is known that certain mycotoxins (ochratoxin A and B, OTA and OTB; fumonisin B_1, FB_1; citrinin, CIT) which occur in human foods cause tumors in laboratory rodents, but data on their genotoxic effects in human cells are scarce. In most conventional in vitro genotoxicity tests mainly negative results were obtained with these compounds. Therefore we studied the effects of these fungal toxins in a human derived hepatoma cell line (HepG2) with the micronucleus (MN) and the single cell gel electrophoresis (SCGE) assay. These cells retained the activity of phase I and phase II enzymes. Additionally, also bacterial (Salmonella/microsome) assays were carried out with enzyme homogenate prepared from HepG2 cells. In MN assays clear dose-dependent effects were found with OTA (≥5 µg/ml), CIT (≥2.5 µg/ml) and FB_1 (≥25 µg/ml). Also in SCGE experiments positive effects were seen with OTA and FB_1, but not with CIT. Subsequent experiments with pancentromeric probes showed that the MN induction of CIT is due to aneugenic effects which cannot be detected in SCGE assays. Bacterial mutagenicity tests with and without enzyme homogenate from HepG2 cells (which contains only active phase I enzymes) failed to detect mutagenic effects of the mycotoxins. OTB was devoid of activity in all three tests. Our results show that the different fungal toxins cause DNA-damage in human derived cells. Therefore consumption of mouldy foods might lead to DNA damage and increased cancer risk in humans.

L8

Application of DNA Microarrays for Immunotoxicological Testing

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The goal of our present research is the development of a DNA microarray as an in vitro toxicological test system to reveal the sensitising potential of chemicals. Dendritic cells from human donors are used to analyse gene expression after treatment with different potential allergens. We developed a DNA microarray containing 65 immune genes, housekeeping genes, negative controls and external spike controls for normalization. Different labelling techniques were tested, especially taking into account the limited amount of immune cells and RNA available. THP-1 cells were stimulated with LPS and gene expression in LPS-treated cells was compared to gene expression in untreated cells. Applying a direct labelling protocol, increased expression of several immune relevant genes could be shown with either 10 µg or only 2 µg of total RNA. However, more upregulated genes could be detected with an indirect labelling method, again using only 2 µg of RNA. Based on these results, we now use the indirect labelling protocol. In order to find candidate genes for the identification of immunotoxic chemicals, different model allergens are applied to dendritic cells and gene expression is compared to gene expression in untreated cells. To establish a general treatment procedure, expression patterns at different time points after application of allergens are compared. Our preliminary results indicate that the microarray technology could provide an in vitro alternative to animal test methods currently available to predict the sensitizing effects of chemicals.

L9

The Prediction of Respiratory Sensitizing Potential of Chemicals in a Modified Local Lymph Node Assay

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The local lymph node assay (LLNA) is a validated method to identify chemical allergens. The test substance is applied topically and the immunogenic potential of the chemical is evaluated by the proliferative response of T cells in the draining lymph nodes. Though the skin is known to be the relevant route of exposure for contact sensitization, experiments have shown that, at least in rodents, dermal application of chemical allergens may also be relevant for the development of respiratory sensitization. Cytokine profiling has been suggested to differentiate between contact and respiratory sensitizers in the LLNA. The dichotomy of T helper cells is a well known phenomenon. Naïve T cells either differentiate to TH1 or

TH2 cells characterized by their specific cytokine profile. TH2 cells promote humoral immune function while TH1 cells promote DTH reactions. We used a slightly modified LLNA and a cytometric bead array for the determination of cytokines. Flow cytometric bead arrays consist of bead populations with discrete fluorescence intensity, conjugated with capture antibodies against a set of cytokines. The fluorescence measurement of a second antibody allows the quantitative analysis of cytokines in the test sample. Three contact, three respiratory sensitizers and the irritant SDS have been tested. No difference in TH1 cytokines after ex vivo ConA stimulation was found, but in all animals a high amount of interleukin-4 was induced with respiratory compared to contact sensitizers, SDS or the solvent control thus allowing a clear identification of the sensitizer.