OXYGEN CLUB OF CALIFORNIA

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OXIDANTS AND ANTIOXIDANTS IN BIOLOGY

BOOK OF ABSTRACTS

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FESS PARKER'S DOUBLE TREE RESORT

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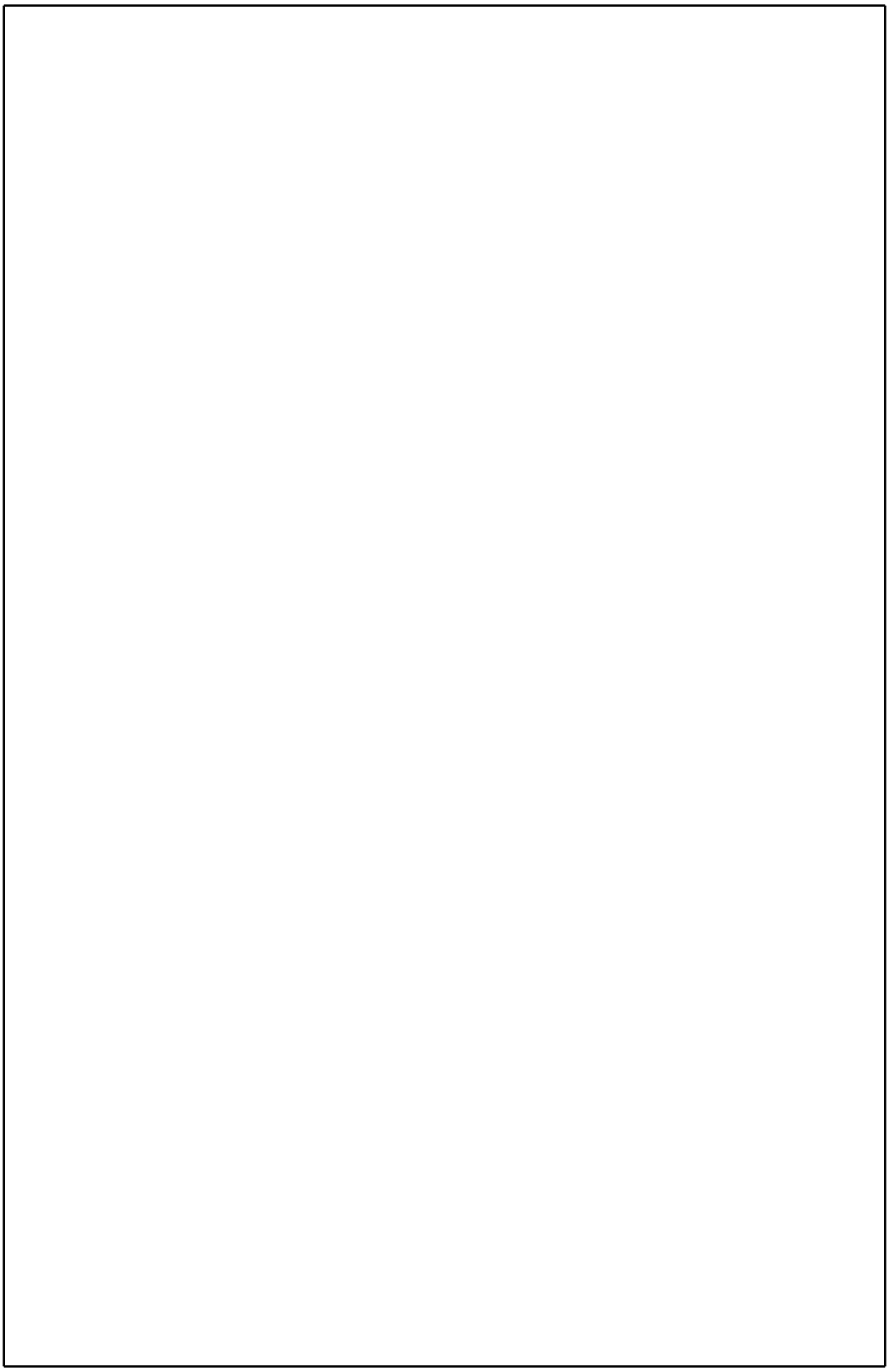

KEYNOTE LECTURE

Cancer-Associated Mutations in the Manganese Superoxide Dismutase Gene: Lung versus Prostate

JOE M. MCCORD

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The relationship between oxidative stress and carcinogenesis has long been recognized, but it is by no means consistent when comparing cancers of different types from various tissues. Epidemiological data suggest a strong relationship between oxidative stress and prostate cancer, for example, but a similar relationship is less obvious for breast cancer. Much attention has focused on the mitochondrial manganese-containing superoxide dismutase, SOD2. Oberley and colleagues have reported that SOD2 activity is nearly always decreased in transformed cells, but the mechanisms responsible for the decrease have not been fully delineated. We have found coding mutations that destabilize SOD2 in leukemia and lung cancer, but these are relatively rare. Much more common are three promoter mutations first described by St. Clair and colleagues. We find at least one of these mutations in 13 of 13 prostate cancers and in many but not all lung cancers. While the promoter may be responsible for "normal" metabolic regulation of SOD2, an enhancer in intron 2 appears to confer responsiveness to inflammatory cytokines via C/EBP and NF-kappa B sites. We have found two new enhancer mutations in prostate cancers that disrupt C/EBP sites. Thus, failure of normal metabolic regulation of SOD2 may predispose certain tissues to cancer, while other tissues may be placed at greater risk if they lose cytokine inducibility of SOD2.

SESSION I
NITRIC OXIDE INTERACTIONS WITH MEMBRANES

Physical and Chemical Dynamics of NO in Membranes

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Both NO and O_2 are more soluble in hydrophobic solvents than in aqueous solution. This means that in a heterogeneous system such as tissues, NO and O_2 will selectively partition into hydrophobic phases such as cell membranes, lipoproteins, chylomicrons, and even the hydrophobic interior of aqueous proteins. Mathematical modeling reveals that for the third-order reaction of NO with oxygen ($2NO + O_2 \ddagger$ products), there should be a dramatic acceleration of this reaction within the hydrophobic phase from simply the concentrating effect of the partitioning of the reactants. This hypothesis has been tested using aqueous solutions of detergent micelles, phospholipid vesicles, and also biological membrane vesicles. Acceleration of NO autoxidation increases with increasing volume of hydrophobic phase. The acceleration is relatively insensitive to the chemical identity of the phase (e.g., cationic, anionic, or zwitterionic detergent), and only occurs with detergents above the critical micellar concentration (CMC). These results indicate that the effect is due to hydrophobic partitioning and thus concentration within the hydrophobic phase. Modeling suggests that the reaction $2NO + O_2 \ddagger$ products occurs approximately 300x faster within the hydrophobic phase (on a per unit volume basis) than in the aqueous phase, for a system where the hydrophobic volume is substantially smaller than the aqueous phase volume. These results suggest that hydrophobic tissue phases act as a “lens” to concentrate and focus the reaction of NO with O_2, and thus is an important site of nitrosative activity.

Interactions of peroxynitrite and nitric oxide with mitochondria

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The interactions of peroxynitrite and nitric oxide with mitochondria are of interest in both physiological and pathophysiological situations. To investigate these processes we have developed mitochondrially-targeted reagents. Among these are mitochondria-specific antioxidants that block some of the damaging effects of peroxynitrite. In addition, as both nitric oxide and peroxynitrite can modify mitochondrial protein thiols, we have developed mitochondrially targeted thiol reagents. These have been used to investigate the status of mitochondrial thiols in cells following exposure to nitric oxide and peroxynitrite. I will describe some of our recent work using these reagents to investigate the interaction of mitochondria with nitric oxide and peroxynitrite.

References

Selective targeting of a redox-active ubiquinone to mitochondria within cells: antioxidant and antiapoptotic properties. Geoffrey F. Kelso, Carolyn M. Porteous, Carolyn V. Coulter, Gillian Hughes, William K. Porteous, Elizabeth C. Ledgerwood, Robin A. J. Smith and Michael P. Murphy. *J. Biol. Chem.* (2001) In press

Mitochondrially targeted antioxidants and thiol reagents. Carolyn V. Coulter, Geoffrey Kelso, Tsu-Kung Lin, Robin A. J. Smith and Michael P. Murphy. *Free Rad.l Bio. Med.* (2000) **28** 1547-1554

Selective targeting of an antioxidant to mitochondria. Robin A. J. Smith, Carolyn M. Porteous, Carolyn V. Coulter and Michael P.
Murphy. *European J. Biochem.* (1999) **263** 709-716

Nitration and oxidation of a hydrophobic tyrosine probe by peroxynitrite in membranes: comparison with nitration/oxidation of tyrosine by peroxynitrite in aqueous solution.

HAO ZHANG, JOY JOSEPH, JIMMY FEIX, NEIL HOGG AND BALARAMAN KALYANARAMAN*

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It has been reported that peroxynitrite will initiate both oxidation and nitration of tyrosine, forming nitrotyrosine and di-tyrosine, respectively. We compared peroxynitrite-dependent oxidation and nitration of a hydrophobic tyrosine analog in membranes and tyrosine in aqueous solution. Reactions were carried out in the presence of either bolus addition or slow infusion of peroxynitrite, and also using the simultaneous generation of superoxide and nitric oxide. Results indicate that nitration of the hydrophobic tyrosyl probe located in a lipid bilayer was significantly greater than its oxidation to the corresponding dimer. During slow infusion of peroxynitrite, nitration of the membrane-incorporated tyrosyl probe was greater than that of tyrosine in aqueous solution. Evidence for hydroxyl radial formation from decomposition of peroxynitrite in aprotic solvents was obtained by electron spin resonance spin trapping. Mechanisms for nitration of the tyrosyl probe in the membrane are discussed. We conclude that nitration but not oxidation of a tyrosyl probe by peroxynitrite is a predominant reaction in the membrane. These findings differ from those previously reported for peroxynitrite-dependent oxidation/nitration of free tyrosine in aqueous solution (Pfeiffer, S., Schmidt, K., and Mayer, B. (2000) *J. Biol. Chem.* **275**, 6346-6352). Thus, the local environment of target tyrosine residues is an important factor governing its propensity to undergo nitration in the presence of peroxynitrite.

The Mitochondrial Nitric Oxide Synthase

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The mitochondrial production of nitric oxide (NO) was recognized a few years ago simultaneously by Ghafourifar and Richter (1997) and by Giulivi et al. (1998). Production of NO by mitochondrial membranes is usually assayed spectrophotometrically by metHb or metMb formation from HbO_2 or MbO_2 at 577-591 nm; by EPR through metal-NO complex formation of specialized probes; and by ^{3}H-citrulline formation and liquid scintillation counting. Mitochondrial nitric oxide synthases (mtNOS) of liver, thymus and thyroid have a MW of 130 kDa and react with the antibody against iNOS. Brain mtNOS has a MW of 144 kDa and reacts with the antibody against the C-terminal segment of nNOS, and does not react with the antibody against the N-terminal segment of nNOS. The rate of NO production in air saturated buffers, usually 0.3 to 0.6 nmol NO/min.mg protein, can be estimated as 0.1-0.2 nmol NO/min.mg protein at physiological O_2 tissue levels, accounting in such case for about 0.2 % of organ O_2 uptake. The measured Km O_2 values for liver, kidney and brain were 15, 24 and 68 μM O_2. The continuous production of NO maintains an intramitochondrial steady state level of about 50-100 nM NO. There are an oxidative utilisation pathway, through the formation of ONOO- after a termination reaction with O_2-, that accounts for 60 % of NO production, a reductive utilisation pathway with formation of NO- by ubiquinol reduction that accounts for 20 % of NO production, and diffusion to the extra-mitochondrial space that accounts for the remaining 20 %. The steady state level of 50-100 nM NO should inhibit cytochrome oxidase activity by 20-30 %, acting competitively with O_2 at tissue levels. Isolated mitochondria

and cells increase their rates of O_2 uptake by 20-30 % when supplemented with NOS inhibitors such as L-NMMA or nitro-arginine. Increase of NO production in isolated thymus mitochondria is recognized with a t1/2 of 15 min after supplementation with the apoptosis inducers etoposide, methylprednisolone and thapsigargin; this effect precedes cytochrome c release and the loss of respiratory control that show $t_{1/2}$ of 30 min. A series of drugs administered in vivo have shown the effect of modifying mtNOS activity in the isolated mitochondria; haloperidol and chloropromazine decrease brain mtNOS activity by 50-60 %; thyroxine (T4) decrease liver mtNOS by 65 %; and enalapril and losartan increase liver and heart mtNOS by 50-100 %.

Redox regulation of NO biochemistry by hemoglobin

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The reactions between Hemoglobin (Hb) and nitric oxide (NO) have been intensely studied recently both from the perpective of physiological mechanisms of regulation of blood flow and development of Hb-based blood substitutes. Novel insights suggest that both physical properties associated with the red blood cell membrane and NO interactions with reactive thiols on the Hb molecule are involved in preserving and promoting NO-dependent vasodilation in vivo. In the latter case, S-nitrosohemoglobin (SNOHb) has been suggested to mediate vessel relaxation through mechanisms that remain controversial and unclear. In this presentation data will be presented that suggest roles for distinct redox states of NO as mediators of SNOHb bioactivity. The potential regulatory role of the red blood cell membrane in these processes and development of blood substitutes will be discussed.

The impact of the interface between endothelium and erythrocyte on NO signaling.

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Nitric Oxide (NO) produced by the endothelium diffuses both into the lumen and to the smooth muscle cells according to the concentration gradient in each direction. The extremely high reaction rate between NO and hemoglobin (Hb), k_{Hb}= 3-5x10^{7} M^{-1} s^{-1}, suggests that most of the NO produced would be consumed by Hb in the red blood cells (RBCs), which would then block the biological effect of NO. Therefore, specific mechanisms must exist under physiological conditions to reduce the NO consumption by RBCs, in which the Hb concentration is very high (24 mM heme). Using isolated microvessels as a bioassay, we show that physiological concentrations of RBC in the presence of intravascular flow does not inhibit NO-mediated vessel dilation, suggesting that RBCs under this condition are not an NO scavenger. On the other hand, RBCs (50% hematocrit) without intravascular flow reduce NO mediated dilation to serotonin by 30%. In contrast, free Hb (10 μM) completely inhibits NO-mediated dilation with or without intravascular flow. The effect of flow on NO consumption by RBCs may be attributed to the formation of an RBC-free zone near the vessel wall, which is caused by hydrodynamic forces on particles. Intravascular flow does not affect the reaction rate between NO and free Hb in the lumen, because the latter forms a homogeneous solution and is not subject to the hydrodynamic separation. However, intravascular flow only partially contributes to the reduced consumption of NO by RBCs, since without the flow, the NO consumption by RBCs is already about three orders of magnitude slower than free Hb.

NO in regulation of ion channel function

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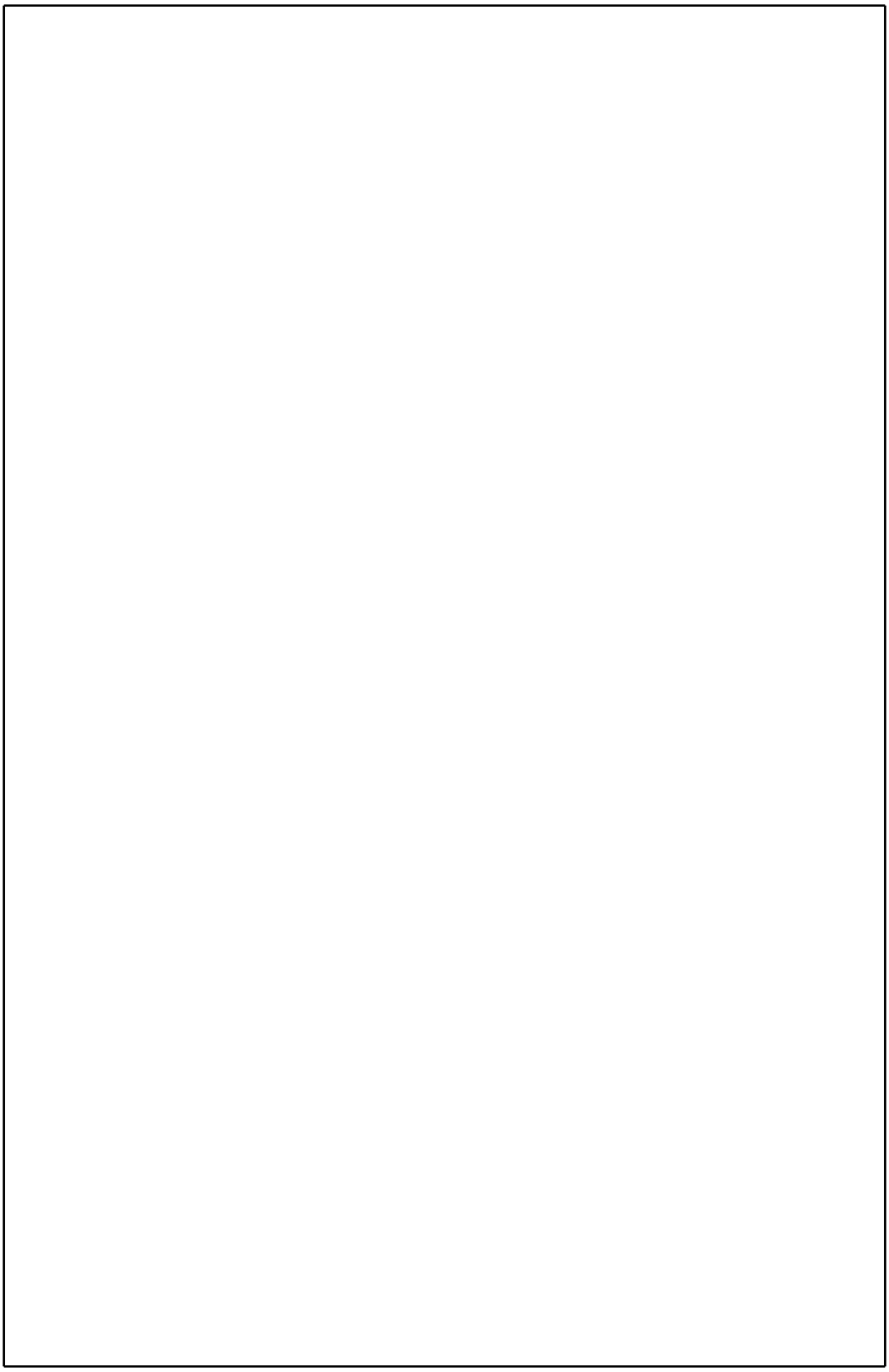
There is growing evidence that NO is involved in regulation of ion transport through biological membranes. The effects of NO on activity of cation channels have been extensively studied.

We have investigated the role of NO in controlling anion flux in human airway epithelial cells. Anions, such as Cl- or HCO3-, play important roles in lung defence mechanisms by regulating the viscoelastic properties of airway surface fluid (ASL). Pathologic alterations of anion transport through CFTR (cystic fibrosis transmembrane conductance regulator) and other channels alter quantity and composition of ASL and contribute to the pathogenesis of chronic inflammatory lung disorders.

Anion transport was monitored using perforated whole cell patch clamp technique in A549 human lung cancer cells. Basal anion flux was reduced following treatment with NOS inhibitors, an effect reversed by NO donors. Moreover, NO donors greatly increased anion transport. These effects of NO were cGMP-dependent. Exposure of cells to cytokines resulted in expression of large amounts of iNOS and formation of ONOO- that were associated with reduced conductance of anions. Moreover, large amounts of exogenous ONOO- impaired anion channel function. NO donors did not increase anion flux following cytokine treatment. These detrimental effects of cytokines were reduced by selective inhibition of iNOS with 1400W. Thus, NO controls anion transport in normal airway epithelium. Excessive generation of NO and ROS during inflammation may affect proteins of anion channels leading to altered composition of ASL thus contributing to the pathogenesis of acute and chronic obstructive lung disorders.

Supported by Canadian Institutes of Health Research, Cystic Fibrosis Foundation of Canada and Alberta Heritage Foundation for Medical Research.



SESSION II

MYELOPEROXIDASES AND OTHER PEROXIDASES IN CARDIOVASCULAR DISEASE

Identification of structurally specific oxidized phospholipids as ligands for the macrophage scavenger receptor CD36

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Recent studies suggest that myeloperoxidase (MPO) and the macrophage scavenger receptor CD36 each play important respective roles in the generation and uptake of oxidized forms of LDL, leading to lesion development and atherosclerosis. We now identify a novel class of specific oxidized phospholipids generated by MPO that serve as high affinity ligands for CD36. Synthetic homogeneous phosphatidylcholine (PC) molecular species were exposed to the MPO-H_2O_2-NO_2- system of monocytes and products were separated by multiple sequential HPLC. Lipids were analyzed by on-line electrospray-ionization tandem mass-spectrometry (LC/ESI/MS/MS) and for their ability to bind to CD36. Four major structurally related oxidation products with CD36 binding activity were identified from 16:0, 20:4 PC, and four corresponding analogs were identified as the major active species following oxidation of 16:0, 18:2 PC vesicles. Synthetic standards were generated for each lipid, their structures confirmed by 1H and 13C NMR, and then used to recapitulate both CD36 binding activity and the expected LC/ESI/MS/MS characteristics as free lipids and following derivatization. Anti CD36 blocking mAb and functional CD36-GST-fusion protein specifically inhibited binding of identified ligands to CD36. Incubation of CD36-transfected cells with DiO-labeled unilamellar phospholipid vesicles containing identified ligands produced patterns of staining characteristic for receptor-mediated endocytosis. Based upon the identified structures, and structure function studies with multiple distinct synthetic analogs, we have defined the core functional groups required for high affinity saturable binding to CD36 as sn-2 γ-hydroxy(oxo)-α,β-unsaturated carbonyl-containing PC. Finally, LC/ESI/MS/MS studies confirm these lipids are formed *in vivo*.

Peroxidase-Based Mechanisms for Impaired Nitric Oxide Signaling During Inflammation

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Myeloperoxidase (MPO) is an abundant heme protein of mammalian phagocytes that is typically perceived to play prominent roles in host defense and inflammatory tissue injury. While its microbicidal functions are well established *in vitro,* humans deficient in MPO are not at unusual risk for infection. These 'experiments of nature' question the primary role of MPO in host defense, and suggest that MPO manifests additional and/or alternative functions. Here we show that MPO is a catalytic nitric oxide (NO) oxidase that modulates vascular NO signaling during inflammation. This activity of MPO is hydrogen peroxide (H_2O_2)-dependent, with the physiologic mechanism of NO consumption largely mediated by reaction of NO with substrate radicals (ie. tyrosyl and ascorbyl radicals) generated by MPO. The capacity of MPO to act as a competitive modulator of NO bioavailability in the vasculature is facilitated by its rapid heparin/heparan-dependent transcytosis across endothelial cells. Transcytotic migration of MPO into rodent vascular tissue, both *in vitro* and *in vivo*, diminishes NO-dependent signaling and vasomotor function. In support of this tenet, mice deficient in MPO were found to be resistant to impaired NO-mediated vascular relaxation in an acute model of sepsis. Given the highly conserved structure and catalytic activities of heme peroxidases, these results reveal a novel paradigm for the regulation of NO-dependent signaling pathways in a diverse range of organisms and physiological contexts.

Myeloperoxidase deficiency does not inhibit atherosclerosis in mice.

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Myeloperoxidase (MPO), a heme enzyme secreted by activated phagocytes, generates an array of oxidants proposed to play critical roles in host defense and local tissue damage. To investigate the actions of MPO *in vivo*, we used gene targeting to generate MPO-deficient mice. Neutrophils from homozygous mutants lacked peroxidase and chlorination activity *in vitro* and were impaired in their ability to kill *C. albicans in vivo*. To examine the potential role of MPO in atherosclerosis, a LDL receptor-deficient mouse model was utilized. LDL receptor-deficient mice were lethally irradiated, repopulated with MPO-deficient or wild-type bone marrow and fed a high fat, high cholesterol diet for 14 weeks. White cell counts and plasma lipoprotein profiles were similar between the two groups at sacrifice. Cross-sectional analysis of the aorta indicated that lesions in MPO-deficient mice were about 50% larger than controls (n=21 per group, p=0.0003). Similar results were obtained in a genetic cross with LDL receptor-deficient mice. The mechanism by which this is occurring is not known. In contrast to human atherosclerotic tissue where MPO and its reaction products are found, neither MPO nor chlorotyrosine were observed in mouse atheroma. These data suggest an unexpected, protective role for MPO-generated reactive intermediates in murine atherosclerosis and highlight the importance of considering species differences when using animal models.

Exercise lowers cholesterol in LDL receptor deficient mice and may prevent atherosclerosis by inducing arterial antioxidant enzymes.

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Oxidative stress has been implicated in cardiovascular diseases. Exercise, a deterrent of heart disease, also paradoxically induces an oxidative stress. We propose that oxidative stress in the plasma compartment might protect against atherosclerosis by promoting oxidative clearance of LDL from plasma and by inducing antioxidant enzymes in the artery. High fat diet fed, exercise-trained LDL-receptor deficient (LDLr-/-) male mice showed an induction of arterial catalase and nitric oxide synthase. Additionally, they also had lower plasma cholesterol, and a 40% decrease in atherosclerotic lesions in comparison to the sedentary controls. Since LDL receptor-deficient mice were used in this study, it is unlikely that plasma LDL is cleared by LDL-receptor-mediated pathways. Our results would indicate that exercise-induced oxidative stress in plasma might help to oxidize and clear LDL by the liver by scavenger pathways. The induction of antioxidant enzymes in the artery might limit oxidation in the artery and afford protection against atherosclerosis.

Electronegative LDL subfraction (LDL-) modulates of PPARalpha signaling by in endothelial cells

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Elevated LDL- levels are associated with accelerated atherogenesis in familial hypercholesterolemia, diabetes and in hemodialysis patients. Structural and compositional LDL- modifications, including those mediated by hemoglobin radical in blood, markedly change the signaling properties of LDL- compared to native LDL. We found that LDL- activates inflammatory NFκB and AP-1 signaling pathway, particularly in presence of TNFα. This leads to the expression of the adhesion molecule VCAM-1 in human endothelial cells (EC) consistent with the pro-inflammatory properties of LDL-. Activation of nuclear receptor PPARα in presence of its synthetic ligand fenofibrates interferes with NFkB and AP-1 pathways and decreases VCAM-1 expression. We show that the proportion of LDL- in total LDL correlates with the degree of PPARα ligand formation utilizing an LBD-GAL4 screening system. Lipoprotein lipase (LPL) mediated lipolysis of LDL and LDL- lead to a marked increase in PPARα activation (5-30μg/ml) that was similar to that observed with fenofibric acid (100μM). Ectopic LDL receptor over-expression does not increase this PPARα-LBD response, arguing against a receptor-mediated mechanism for this PPARα ligand production. Consequently, in presence of LPL, LDL- decreases VCAM-1 expression. These findings suggest that LPL represents a physiologic pathway for PPARα ligand generation that might limit pro-inflammatory and pro-atherogenic responses of LDL-.

Session III

Plasma Membrane Oxidases: Role in Reactive Oxygen Species-mediated Signaling

Oxygen sensing and oxygen-dependent gene expression

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A growing number of physiologically relevant genes are regulated in response to changes in intracellular oxygen tension. It is likely that cells from a wide variety of tissues share a common mechanism of oxygen sensing and signal transduction leading to the activation of the transcription factor hypoxia inducible factor 1 (HIF-1). There is growing evidence that the oxygen sensor is a flavoheme protein and that the signal transduction pathway involves changes in the level of intracellular reactive oxygen intermediates (ROI). We have recently cloned a novel fusion protein consisting of an N-terminal domain with homology to cytochrome b5 and a C-terminal domain homologous to cytochrome b5 reductase. The expressed protein is a cyanide insensitive NADPH oxidase and therefore a candidate for the oxygen sensor. This flavoheme protein is widely expressed in cell lines and tissues, with localization in the perinuclear space. It utilizes either NADH or NADPH to convert oxygen to superoxide with a half maximal velocity at 2% oxygen. It can also function as a ferric reductase. The activation of HIF-1 by hypoxia is likely to depend upon ROI-dependent rescue of its α-subunit from oxygen-dependent degradation in the proteasome, allowing it to form a heterodimer with HIF-1β (ARNT), which then translocates to the nucleus, enabling transcription of genes whose cis-acting elements contain cognate hypoxia response elements.

Reference: Zhu H, Qiu H, Yoon H-WP, Huang S, Bunn HF. Identification of a novel cytochrome *b*-type NAD(P)H oxidoreductase ubiquitously expressed in human cells Proc. Natl. Acad. Sci. USA 1999, 96:14742-14747.

Sensors, mediators and responses of the oxygen sensing signal pathway

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The heterogeneous oxygen partial pressure (PO_2) distribution in mammalian organs requires for triggering gene expression and ion channel conductivity an O_2-sensing signal cascade with three responses: optimizing of cellular functions during normoxia, adaptation of cell function under hypoxia and survival of cell function to withstand anoxia. It is not clear whether these different responses are induced by various O_2 sensing signal cascades. One signal cascade, however, is supposed to consist of mitochondrial and/or non mitochondrial heme proteins sensing oxygen with a subsequent second messenger formation as for instance reactive oxygen species (ROS) in particular hydroxyl radicals (OH•) which influence transcription factor stability as well as ion channel pore formation. NADPH oxidase isoforms with different gp91phox subunits as well as an unusual cytochrome c oxidase (CYT) with a hemea/ hemea3 relationship of 9:91 as detected by light absorption photometry in the carotid body as well as human liver tumor cells (HepG2) will be discussed as putative oxygen sensor proteins. Whereas CYT might be of special importance for the carotid body NADPH isoforms generating ROS in dependence on PO_2 are considered as more general cellular O_2 sensors. The formation of OH• by a perinuclear Fenton reaction from hydrogen peroxide (H_2O_2) is imaged three dimensionally by 1 and 2 photon confocal laser miscroscopy in HepG2 cells using dihyhdrorhodamine 123 or 2'7'-dicholorodihydrofluorescin as indicators. Hot spots of OH• generation are to be seen predominantly in the endoplasmatic reticulum (ER) but also in mitochondria (MIT). It is concluded, that heme and non heme iron binding proteins in the ER and MIT compartment represent the perinuclear hot spots which degrade H_2O_2 originating from NADPH oxidase isoforms.

The Nox Family of Homologs of gp91phox

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Six human homologs of gp91*phox* have been cloned, and additional homologs have been identified in rodents, C. *elegans* and *Drosophila*. The homologs fall into two major groups, the Nox group [Nox for NAD(P)H oxidase] and the Duox group [referring to Dual oxidase]. Nox1, Nox3, Nox4 and Nox5 are like gp91*phox* around 65 kDa in size, and show 20-60% sequence identity with one another, including conserved putative binding sites for FAD, NADPH and hemes. The Duox group proteins range from 175 to 180 kDa, and contain not only a C-terminal region that is homologous to the Nox proteins, but also an N-terminal region that is homologous to peroxidases and a central region that contains calcium binding EF hands. Nox1, when expressed in NIH 3T3 cells causes cell transformation and confers a highly tumorigenic phenotype which includes both an increased rate of cell division and activation of the angiogenic switch. The phenotype is reversed by co-expression of catalase, indicating that hydrogen peroxide generated secondarily by the enzyme serves as the intracellular transformation/angiogenesis signal. By DNA microarray analysis, Nox1 expression induces changes in a large number of signaling, cell cycle and other proteins, but does not induce a response that is characteristic of an oxidative stress, indicating that the enzyme generates signalling levels of reactive oxygen. The function of the Duox proteins has been investigated using biochemical approaches and RNA interference in C. *elegans.* The peroxidase homology domains of human and C. *elegans* Duox show peroxidase activities, including the cross-linking of tyrosine residues. In C. *elegans*, elimination of Duox expression disrupts the structure of the cuticle, the collagenous extracellular matrix of the worms by preventing cross-linking of tyrosine residues. Thus, in worms, Duox is involved in the biogenesis or stabilization of the extracellular matrix. Thus, novel homologs of gp91*phox* reveal unique biological functions for reactive oxygen.

Superoxide production by phagocytes: regulated assembly of the nadph oxidase and role of the rho gtpase rac2

M.C. DINAUER

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Superoxide production by activated phagocytes is catalyzed by a multi-subunit NADPH oxidase. Genetic defects in the NADPH oxidase result in chronic granulomatuous disease (CGD), an inherited disorder characterized by absent phagocyte superoxide release and recurrent infections. The active oxidase complex contains a membrane bound flavocytochrome b heterodimer that mediates electron transfer and several soluble regulatory proteins, including Rac, a Rho family GTPase. Transgenic expression of wild-type and mutant oxidase subunits in cell lines have established the gp91*phox* subunit of flavocytochrome b as the enzyme redox center and identified other functional domains essential for oxidase assembly and activity. Recent studies have also shown that expression of constitutively active derivatives of Rac is sufficient to activate the NADPH oxidase in a whole cell model. To define the role of the hematopoietic-specific Rac2 isoform, the predominant species in human neutrophils, a Rac2-/- mouse was generated by gene targeting. Superoxide production in Rac2-/- PMN was defective in response to PMA, fMLP, and IgG-opsonized particles, but normal in response to opsonized zymosan. Other functional defects included impaired F-actin polymerization, chemotaxis, and L-selectin -mediated adhesion. An important role for Rac2 in PMN function was further established by the identification of a dominant-negative mutant of Rac2 in an infant who presented with recurrent pyogenic infections and PMN defects similar to those found in Rac2-/- mice. Taken together, these observations provide genetic evidence that Rac2 functions as a critical regulator of the NADPH oxidase and other functional responses of neutrophils.

Pro-inflammatory cytokines induce p47phox phosphorylation in human neutrophils. Evidence for a new pathway which regulates superoxide anion production.

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Superoxide anion production by neutrophils is essential for host defenses and participates in inflammatory reactions. This process can be potentiated by prior exposure to "priming" agents such as the pro-inflammatory cytokines GM-CSF, TNF and IL-8. We investigated the effects of these cytokines on the phosphorylation of the cytosolic NADPH oxidase component p47phox. Preincubation of neutrophils with GM-CSF, TNF or IL 8 induced phosphorylation of p47phox with the following order of potency: GM-CSF>TNF>IL-8. GM-CSF- and TNF-induced p47phox phosphorylation was time- and concentration-dependent and ran parallel to the priming effect of these cytokines on superoxide production. Two-dimensional tryptic peptide mapping of p47phox showed that GM-CSF and TNF induced phosphorylation of one major peptide. The chemotactic peptide fMLP induced phosphorylation of several peptides, an effect enhanced by GM-CSF and TNF pretreatment. In contrast to fMLP and PMA, GM-CSF- and TNF-induced phosphorylation of p47phox was not inhibited by the protein kinase C inhibitor GF109203X. The protein tyrosine kinase (PTK) inhibitor genistein and the PI3Kinase inhibitor wortmannin inhibited the phosphorylation of p47phox induced by GM-CSF, TNF and fMLP but not PMA. These results show that the priming action of GM-CSF and TNF on the neutrophil respiratory burst involves partial phosphorylation of p47phox and suggest the involvement of a priming pathway regulated by PTK and PI3K but not by PKC.

Involvement of small GTPase Rac1 in activation of hypoxia inducible factor 1 (HIF-1)

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Mammalian cells exhibit many homeostatic responses to hypoxia, including transcriptional activation of genes encoding proteins that function to increase O_2 delivery and allow metabolic adaptation under hypoxic or ischemic conditions.Hypoxia-inducible factor 1 (HIF-1) is a transcription factor that mediates cellular and systemic homeostatic responses to reduced O_2 availability in mammals, including erythropoieisis, angiogenesis, and glycolysis. Hypoxia induces both the protein expression and transcriptional activity of the HIF-1α subunit. However, the molecular mechanisms of sensing and signal transduction by which changes in O_2 concentration result in changes in HIF-1 activity are poorly understood. In this study, we report that the small GTPase Rac1 is deeply involved in the induction of HIF-1α protein expression and transcriptional activity in hypoxic cells.

Proatherogenic Oxidative Signaling in Vascular Cells

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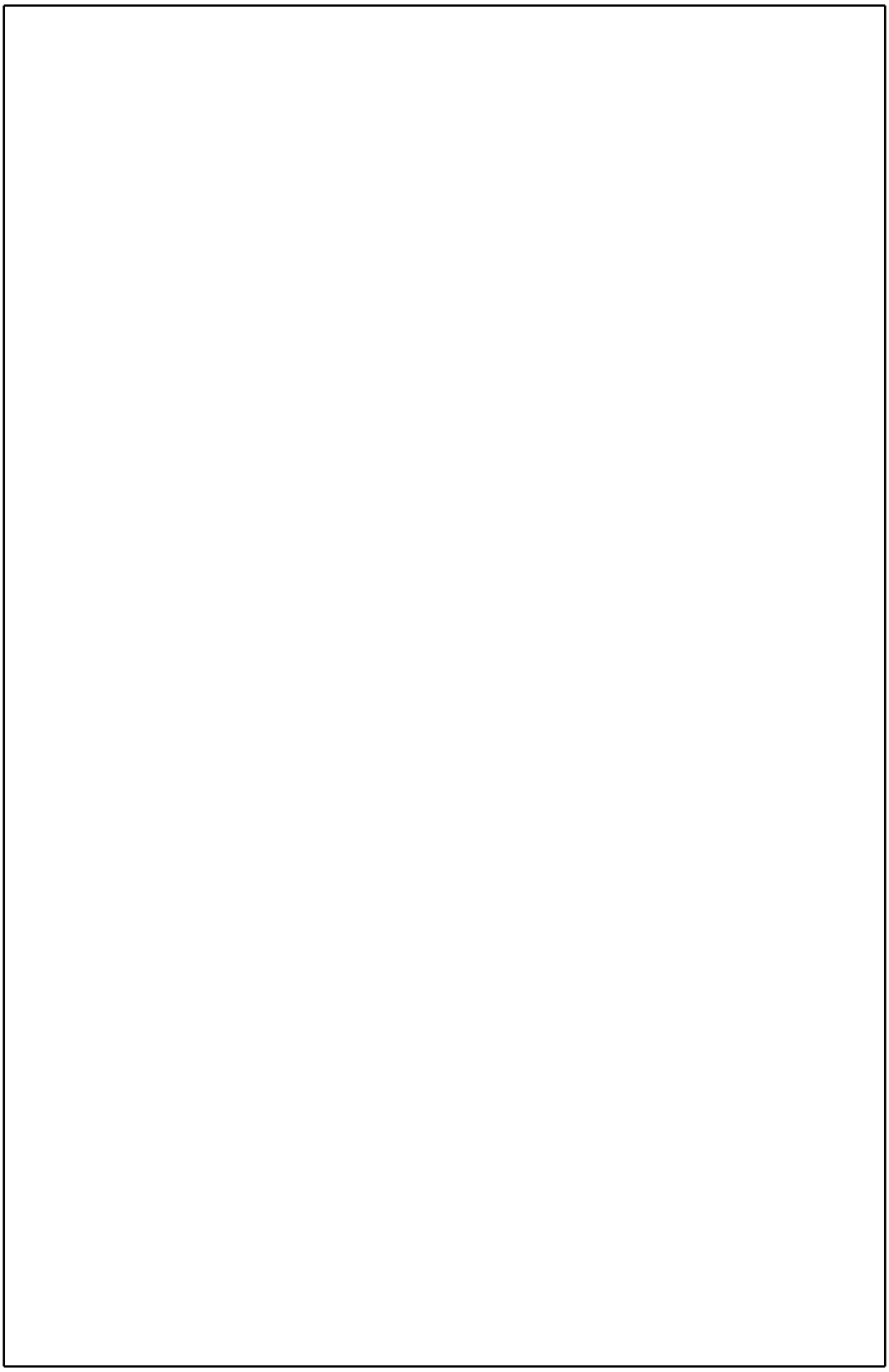
Atherosclerosis is a disease of many risk factors, a complex pathology, and diverse clinical manifestations. Although no unifying pathway has been identified, oxidative signaling pathways meet many of the characteristics of such a pathway. The focus of our work over the past several years has been to examine the contribution of ROS to vascular smooth muscle cell (SMC) growth in vitro and to atherogenesis in vivo. In doing so, we have studied two mechanisms that govern ROS production in SMC. One focus for study has been the NAD(P)H oxidase in SMCs. In contrast to it's counterpart in phagocytes, this oxidase consumes NADH preferentially and generates significantly lower concentrations of ROS at a slower rate –likely because differences in subunit composition. Deletion of one of the components of the NAD(P)H oxidase (p47phox; common to both SMC and phagocytes) in cultured SMC interrupts mitogen-stimulated ROS generation. When p47phox is deleted in vivo, in an ApoE-/- background, atherogenesis is inhibited. Mitochondria are also important sources of cellular ROS production. Although many factors can impact mitochondrial oxidative phosphorylation, the ability to modulate the oxidative milieu in mitochondria, to a significant degree, resides in the expression of manganese superoxide mutase (MnSOD). Deficiency in MnSOD in cultured cells leads to mitochondrial damage, decreased ATP production and increased ROS production. MnSOD deficiency in vivo (also in the ApoE-/- background) accelerates atherogenesis. Clearly we have much to learn, for regardless of how ROS are generated, it is easy to overwhelm their production with reducing agents and antioxidants in experimental studies. The crux of the oxidative paradox of atherosclerosis is that data regarding the use of antioxidants for prevention of atherosclerosis do not presently support their use in cardiovascular diseases. Of the many possibil-

ities, and similar to the situation with hypertension or hypercholesterolemia, increased oxidative stress may be a risk factor for only a subset of patients with atherosclerosis, albeit a subset we have no easy way to identify at present. Measuring markers of oxidative injury, such as circulating lipid peroxides or mitochondrial DNA damage (which will be discussed) are promising, but as yet unproven, means to identify patients who might benefit from antioxidant therapy.



Session IV
Protein Oxidation and the Protective Role of Proteolytic Enzymes in Health and Disease

Proteolytic pathways and cellular protection during oxidative stress, aging and disease

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In recent years protein oxidation has been recognized as a normal consequence of aerobic metabolism, and as a potentially cytotoxic outcome of oxidative stress. Proteins undergo direct oxidation, and form adducts with oxidized lipids, carbohydrates, and nucleic acids. Various proteolytic systems protect cells, tissues, and bodily fluids against the accumulation of oxidatively damaged proteins. These include the proteasome in cell cytoplasm, nucleus, and endoplasmic reticulum; the Lon protease in the mitochondrial matrix; and various lysosomal proteases involved in degrading organelles. Under most conditions these proteolytic systems appear to be able to degrade oxidatively denatured proteins as they form. An oxidatively modified protein may contain only a few damaged residues, and the majority of amino acids will be reutilized for protein synthesis. During oxidative stress the rate of protein oxidation can exceed the capacity of the proteolytic systems, and damaged proteins may begin to accumulate. Oxidatively modified proteins often have externalized hydrophobic patches which make them form aggregates. Further reactions can lead to the formation of covalently cross-linked residues; such as dityrosine. Protein aggregates are difficult substrates for most proteolytic enzymes, and cross-linked aggregates may become completely resistant to proteolysis. Such conditions are cytotoxic and can lead to apoptosis or necrosis. Recent experiments suggest that advanced aging and certain diseases may be associated with both a significant increase in cellular oxidative stress (from dysfunctional mitochondria), and a significant decrease in proteolytic capacity. These two coincident events may contribute to the formation of various cellular inclusion bodies, lipofuscin, and ceroid bodies, and to progressively diminishing cellular function.

Relationship between protein oxidative damage and age-related attrition of cellular functions

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A major prediction of the oxidative stress hypothesis of aging is that the rate of accrual of macromolecular oxidative damage is directly linked to the rate of aging of the organism. In this context, oxidative damage to proteins can be suggested to be crucial because oxidized proteins lose function and undergo preferential degradation. Notwithstanding, it has been convincingly demonstrated that most of the enzymes do not exhibit an age-associated decline in their specific activity, which would, seemingly, contradict the widely-held view that molecular oxidative damage during aging is random and ubiquitous. Addressing this issue, our studies on the mitochondria from the flight muscles of flies have indicated that protein oxidative damage during aging and in response to hyperoxia is highly selective involving relatively few proteins. The damaged proteins exhibit corresponding losses in catalytic activity and, perhaps most importantly, experimental extensions in the life span of the flies were found to retard the rate of accrual of oxidative damage to the mitochondrial proteins. The rate of accrual of oxidative damage was directly associated with the rates mitochondrial H_2O_2 generation and oxygen consumption of the flies. We postulate that accrual of oxidative damage and consequent loss of function of selective proteins constitute a mechanism linking oxidative stress and the age-related attenuation of cellular functions. (This research was supported by grants from N.I.H. – N.I.A.)

Selective recognition and degradation of oxidized proteins by the proteasome

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Proteins exposed to oxidative stress are degraded via proteolytic pathways. In the present studies we undertook a series of *in vitro* experiments to establish a correlation between the structural changes induced by oxidation of proteins and the proteolytic rate found upon exposure of the modified protein towards the isolated 20S proteasome.

The model protein RNase A was mildly oxidized by hydrogen peroxide and analyzed by Fourier transformed infrared (FT-IR) spectroscopy. We found strong experimental evidence for oxidation induced conformational rearrangements of the model protein RNase A, and at the same time, for covalent modifications of amino acid side chains. Proteasome digestion measurements on oxidized RNase A revealed a specific time and H_2O_2 concentration dependence. Based on these experimental findings, a correlation between structural alterations detected upon RNase A oxidation and proteolytic rates of RNase A is established.

In a further set of experiments various proteins were used and strongly oxidized. Oxidation was accompanied by the formation of protein aggregates, which were able to inhibit the proteasome in vitro. The inhibition was due to a direct proteasome-protein aggregate interaction. In several neurodegenerative diseases and the aging process the interaction of the proteasome with oxidized protein aggregates seems to be important for the intracellular proteasome activity.

Deficiency of 20S proteasome functions induced by environmental and pathological factors: from structural analysis to pharmacoproteomics

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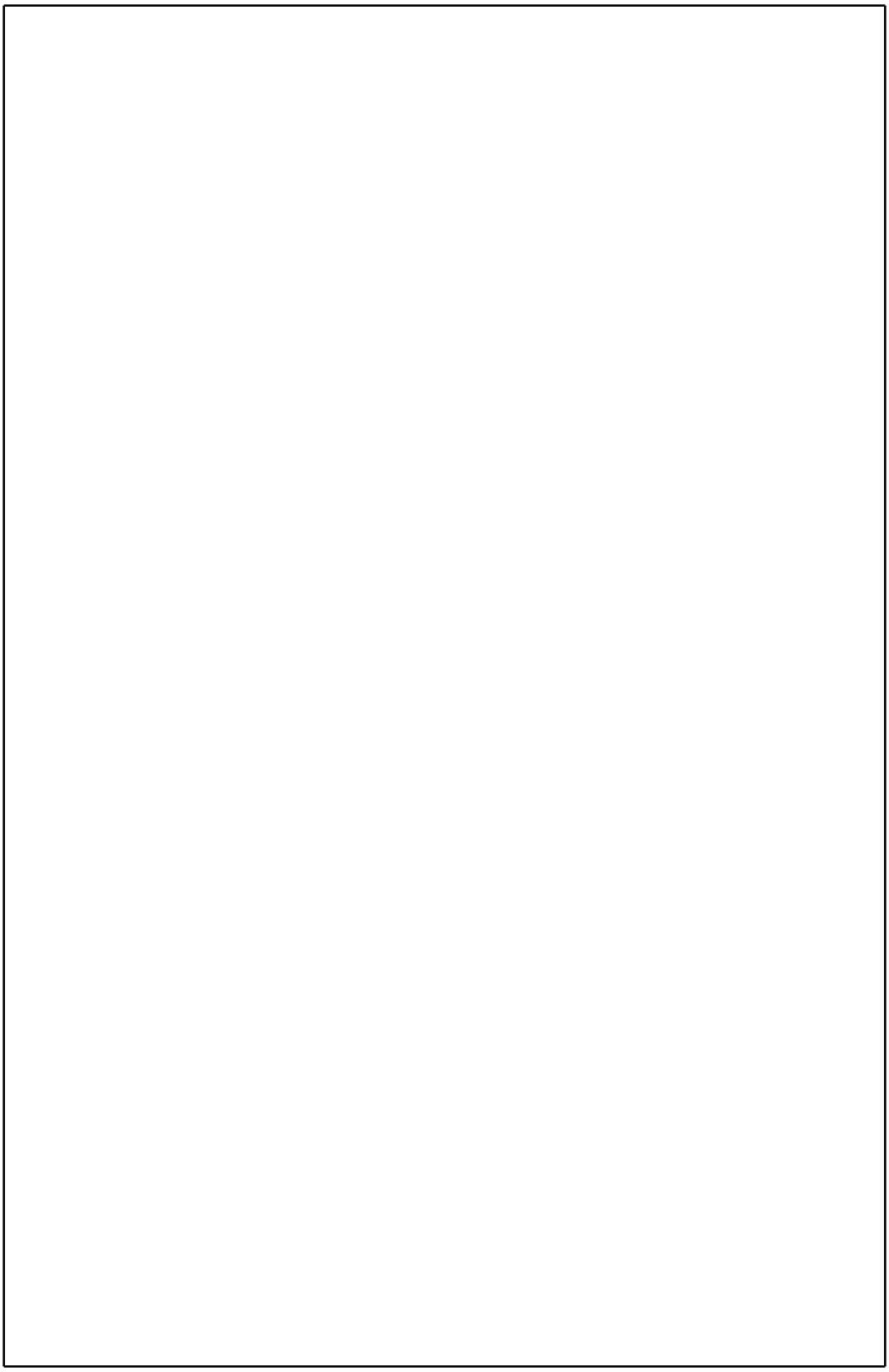
The proteasome is an ubiquitous multicatalytic complex, present in the cytoplasm and the nucleus of all eukaryotic cells, involved in the degradation of most cellular proteins. The proteasome is a key component of the ATP-dependent proteolytic pathway: it allows the regulation of intracellular proteins content, clears the cell of misfolded or nonfunctional proteins, and plays a major role in the processing of major histocompatibility complex (MHC) class I-restricted antigenic peptides. The 20S proteasome has a cylindrical structure made of four stacked rings (2 α and 2 β), each ring composed of 7 subunits, assembled in the order αββα. The 2 inner rings are composed of 7 β subunits and form the proteolytic core. Only 3 β subunits (β1, β2 and β5) display a catalytic activity (post-glutamyl peptide hydrolysing (PGPH), chymotrypsin-like, and tryptic-like activity respectively).

Our current work aims to determine the impact of environmental or pathological factors on the 20S proteasome function. The proteolytic activity of the 20S proteasome can be altered by various environmental factors (stress, cytokines, nitric oxyde,…), pathological states, aging or pharmacological agents. Structural modifications, such as replacement of the catalytic subunits β1, β2 and β5 by the IFN-γ-inducible subunits β1i, β2i and β5i, post-translationnal or chemical modifications, lead to altered activities and modified protein degradation patterns. We will present here our recent progress in structure-function relationship studies on tumor antigen processing by purified 20S human proteasome as well as the biological and structural basis of the proteomics approach developed in our laboratory.



SESSION V

OXIDATIVE STRESS AND ITS ROLE IN CELL REGULATION AND DISEASE

Antioxidant functions of thioredoxin and glutaredoxin systems

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Selenium is an essential trace element with known antioxidant properties. Thioredoxin reductases from mammalian cells are dimeric FAD enzymes built from a glutathione reductase elongated with 16 residues including a conserved carboxyterminal sequence, Gly-Cys-SeCys-Gly, where Se-Cys is selenocysteine. The active site of the enzyme is a selenenylsulfide bridge formed from the conserved Cys-SeCys sequence. In the reduced enzyme electrons are transferred from the redox active disulfide of the other subunit to form a selenolthiol which is the active site in reduction of thioredoxin and peroxides. An unpublished structure of the enzyme at 3 Å supports the model of the enzyme and also shows the open active site with binding of thioredoxin without domain rotations as in the *E. coli* enzyme which is smaller and entirely different. The hydrogen peroxide reductase activity of mammalian thioredoxin reductase is stimulated 15-fold by 2 µM ebselen. The results demonstrate that the antiinflammatory selenazol ebselen, which has weak GSH-peroxidase activity and is used in clinical trials against stroke has major effects via the thioredoxin system and the mechanisms will be described. The solution structure of *E. coli* Grx 2 shows surprising similarities to a family of novel large glutaredoxins with a glutathione-S-transferase-like structure. The implications of this will be discussed in relation to redox regulation and antioxidant functions via thiol redox control and reversible protein glutathionylation.

Anti-inflammatory activity of soluble thioredoxin: redox modulation of chemotactic activity

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Thioredoxin (Trx) is a small (12 kD) redox-active protein with a variety of biological functions. We have reported that intravenous administration of recombinant human Trx can attenuate ischemic reperfusion injury in animal models. Although the possible mechanism of this protective effect of Trx has been considered to be dependent on the anti-oxidant effect of Trx possibly in cooperation with peroxiredoxin, we propose here that an additional mechanism may explain the anti-inflammatory effect of Trx. In a mouse air pouch model, intravenous administration of recombinant human Trx can suppress the LPS-induced down regulation of CD62L on neutrophils, which is an initial event of neutrophil activation prior to adhesion on endothelial cells and extravasation. Moreover, Trx can block the adhesion of neutrophils on endothelial cells and neutrophil extravasation in the air pouch. The active site of Trx is necessary for this activity and the oxidized form of Trx shows the comparable effect of the reduced form. Actually Trx can attenuate the leukocyte infiltration-dependent disorders such as the interstitial pneumonia caused by bleomycin or cytokines. These results indicate that circulating Trx inhibits neutrophil migration into the inflammatory site, which may provide us the additional mechanism of the protective and anti-inflammatory effects of Trx.

Nrf2 and Keap1 Regulation of Oxygen Stress Response

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Antioxidant responsive element (ARE) or electrophile responsive element (EpRE) mediates the transcriptional activation of the genes encoding phase II drug metabolizing enzymes and antioxidative stress genes. The ARE/EpRE consensus sequence shows high similarity to NF-E2 binding sequence, a *cis*-acting erythroid gene regulatory element. Based on the observation, we carried out targeted disruption of the *nrf2* gene in mouse and examined the inducible expression by BHA of several detoxifying enzyme genes in the *nrf2*-deficient mouse. The induction of phase II and other drug metabolizing enzyme genes by BHA was significantly affected in the mouse, demonstrating that Nrf2 regulates the inducible expression of phase II enzyme genes. Nrf2 also regulates the expression of antioxidative stress enzyme genes in the mouse. Thus, Nrf2 appears to be essential for the coordinate induction of phase II detoxifying enzymes and antioxidant enzymes, both of which are under ARE regulation. Detailed analysis of the regulatory mechanisms of Nrf2 activity have led us to the identification of a new protein, Keap1, that suppresses Nrf2 activity by specific binding to its evolutionary N-terminal Neh2 regulatory domain. Nrf2 was liberated from Keap1 by electrophlic agents/phase II inducers, suggesting that the Nrf2-Keap1 system acts as a sensor for the xenobiotics and oxidative stresses. The finding that the ARE response is defective in the *nrf2* knockout mice, concomitant with the progress in the analysis of the Nrf2 activation process and its repression by Keap1, would lead to the elucidation of the molecular basis for the phase II enzyme and antioxidant gene induction.

Antioxidant defense in parasitic trypanosomes

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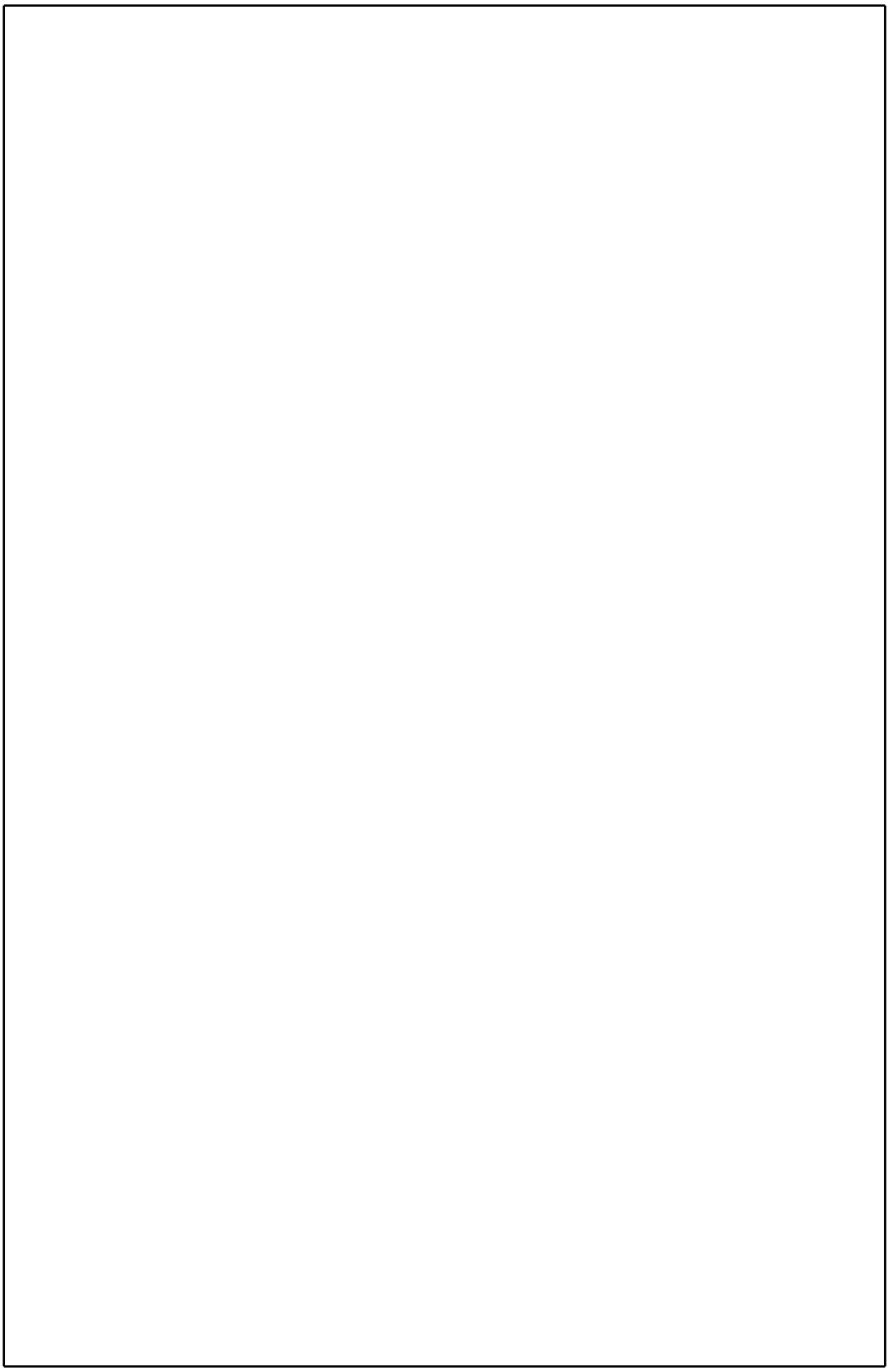
Parasitic trypanosomatids, which comprise the causative agents of life-threatening diseases, are equipped with a unique antioxidant defense system that has been shown to be pivotal for virulence and viability under oxidative conditions. It consists of three oxido-reductases: Trypanothione reductase (TR), tryparedoxin (TXN) and tryparedoxin peroxidase (TXNPx), and the redox mediator trypanothione [bis(glutathionyl)spermidine]. The catalytic mechanisms and specificities of TXN and TXNPx were analyzed by site-directed mutagenesis, molecular modelling and crystallography. TXNPx catalysis involves Cys52 and Cys173. Cys52 is activated by electrostatic interaction of Arg 128 and hydrogen bonding of Thr49). In TXN2 from *C. fasciculata* Cys41 of the 40WCPPCR45 motif is the solvent exposed residue that can attack disulfide bonds in oxidized TXNPx. It is activated by proton shuttling between its SH-group and that of Cys44 which in turn is involved in a network of hydrogen bridges. A productive catalytic complex with trypanothione is achieved by electrostatic interactions with Arg129, Glu73 and Arg45 and hydrogen bonds to the main chain near Ile110 and Cis111. Most of the residues determing substrate binding and, in consequence, specificity, are not conserved in the homologous mammalian thioredoxins. The novel insights into the molecular interactions should improve the chances for successful design of specific inhibitors which may be considered as potential trypanocidal drugs.



Session VI
Role of Antioxidants in Cell Signaling

α -Tocopherol-associated protein(s) (TAPs): structure, function and cellular distribution

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The mechanisms involved in cellular regulation as well as in the preferential cellular and tissue accumulation of α-tocopherol are not yet well established. By using radioactive tocopherol as a tracer we have identified a novel 46 kDa tocopherol associated protein (TAP) in liver cytosol. Cloning into E. coli and in vitro expression of human TAP has been carried out. TAP belongs to a family of hydrophobic ligand binding proteins, which have the CRAL (cis-retinal binding motif) sequence in common. A GTP binding site is also visible in TAP amino acid sequence. The purified recombinant protein binds tocopherol with Kd 4.6 x 10-7 M. TAP mRNA is expressed in all tissues. Data base analyses have shown the existence of three very similar TAP genes now called TAP1, TAP2 and TAP3. Although in different amounts, they are all transcribed in mammalian cells, where they are directed towards different cellular sites.

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Functional Analysis of the α -Tocopherol Transfer Protein in Mice

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The α–tocopherol transfer protein (α-TTP) preferentially maintains plasma *RRR*-α–tocopherol concentrations. However, α–TTP functions in regulating tissue α–tocopherol concentrations are not understood. Additionally, data are lacking on the in vivo preference of α–TTP for natural (*RRR*) and synthetic (*all rac*) α–tocopherols. Therefore, to evaluate in vivo α–TTP functions, a mouse model with a disrupted α–TTP gene (*Ttpa*) was developed (Terasawa et al). *Ttpa*-/- (*n*=5), *Ttpa*+/- (*n*=7), and *Ttpa*+/+ (*n*=3) mice were fed *RRR*-α-[5,7-(C2H3)2]- and *all rac*-α-[5-(C2H3)]-tocopheryl acetates (30 mg each/kg diet) for 3 months. *Ttpa*–/- had exquisitely low plasma and tissue α–tocopherol concentrations compared with *Ttpa*+/+, despite the observation that tissues from all genotypes contained ~85% labeled α–tocopherol. Deuterated natural/synthetic α–tocopherol ratios were 1 in the diet, only 1 in the tissues of *Ttpa*-/-, and nearly 2 in *Ttpa*+/+ tissues (except liver). Thus, α–tocopherol levels are highly dependent upon α-TTP function. Apparently, hepatic α-TTP preferentially selects 2*R*–α–tocopherols from *all rac* α–tocopherol for secretion into plasma. In mice expressing α–TTP, tissue 2*R*–α–tocopherol-enrichment appears to result from uptake from the plasma, which contained twice as much α–tocopherol from natural as from synthetic α–tocopherol. Thus, the observed 2:1 ratio does not support the currently defined international unit of 1.36 natural : synthetic vitamin E (α–tocopheryl acetates).

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α–Tocopherol signaling in endothelial and platelet function

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α-Tocopherol is the principle lipid-soluble antioxidant in human plasma and some studies indicate it may provide cardiovascular protection. To investigate putative mechanisms for α−tocopherol in this regard, the effect of α−tocopherol on vascular function and platelet aggregation has been examined. In animal models of endothelial dysfunction, α−tocopherol improves the activity of endothelium-derived nitric oxide, and this effect is not dependent upon the antioxidant protection of LDL. In fact, α−tocopherol improves endothelial function, in part, due to the inhibition of protein kinase C (PKC) stimulation. This activity of α−tocopherol has been examined in platelets and α−tocopherol inhibits platelet aggregation, in part, through a mechanism that involves PKC. Moreover, the platelet inhibitory activity of α−tocopherol is independent of its antioxidant action, as platelet inhibition is still observed with isoforms of α−tocopherol that are devoid of antioxidant activity. Platelet incorporation of α−tocopherol also has important implications for platelet-derived NO. We have found that platelets nitric oxide bioactivity is enhanced by α−tocopherol and superoxide production is reduced. This effect of α−tocopherol is also associated with a limitation of platelet eNOS phosphorylation. Thus, the α−tocopherol status of both the platelet and the endothelial cell have important implications for NO bioactivity.

Biological significance of carotenoid breakdown products

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Carotenoids are known as micronutrients which have a variety of biological functions. Beside its antioxidant function beta-carotene was reported to have anticarcinogenic effects. As a result the substance is widely used as supplement in food and as prophylactic agent in medicine. However, controversy remains about its potential toxicity after a number of clinical efficacy trials failed. Moreover, there is evidence that under certain conditions derivatives of oxidative breakdown of beta-carotene may exert harmful effects in humans. To study these effects, beta-carotene was bleached with hypochlorite to yield a mixture of breakdown products (BP). Apo-carotenals were already identified as long-chain beta-carotene BP, however, using GC-MS analysis also a number of short-chain products, such as trimethylcyclohexenone, β-cyclocitral, trimethyltetrahydronaphthalene, 5,6-epoxi-β-ionone, dihydroactinidiolide, and methyl-β-cyclocitrylideneacetate, could be identified. To test the potential toxicity Na-K-ATPase activity was assayed in the presence of carotenoid BPs and found to be rapidly and potently inhibited. The effect, mainly due to the aldehydic BPs, was shown also for beta-apo-10'-carotenal and retinal which represent metabolites of enzymatic and non-enzymatic cleavage of beta-carotene. The concentration of the BP mixture that inhibited the enzyme by 50 % (IC50) was equivalent to 10 µM non-degraded beta-carotene, whereas the IC50 for 4-hydroxy-2-nonenal (HNE), a major lipid peroxidation product, was 120 µM. Thus,

carotenoid BPs seem to be more potent inhibitors of Na-K-ATPase than HNE. Mitochondria may be targets of beta-carotene BPs. For investigation of those effects different mixtures of BP were tested: beta-carotene degraded by hypochlorite, retinal, retinal degraded by hypochlorite, beta-ionone, and beta-ionone degraded by hypochlorite. The ADP stimulated oxygen consumption of liver and brain mitochondria was shown to be affected by all beta-carotene BP mixtures: In liver mitochondria 20 µM CBP led to a 30 - 50 %, in brain mitochondria even to a 50 - 60 % decrease of ADP stimulated respiration. The endogenous respiration was hardly affected, but not by all BP mixtures. The mitochondrial SH content was decreased and MDA formation was increased after exposure of mitochondria to beta-carotene BP. Brain mitochondria were more sensitive than liver mitochondria towards effects of beta-carotene BP. The character of the measured inhibitory effects in mitochondrial respiratory parameters argue either for the inhibition of the adenine nucleotide translocator or/and the inhibition of the ATPase by the BP. The results indicate that metabolites derived by oxidation of carotenoids may have a potential toxicity and/or may act as signal modulators, e.g. as initiators of apoptosis in cells.

New insights into vitamin E metabolism

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Side chain degradation to carboxyethyl hydroxychromans (CEHC) has been shown for α-, γ- and δ-tocopherol. CEHCs are excreted in the urine in man and experimental animals, the amounts increasing with increasing intake. The proposed mechanism via ω-oxidation and subsequent ß-oxidation has been supported by the detection of CEHC precursors, α- and γ-CMBHC [(4'-carboxy-4'-methylbutyl) hydroxy-chroman], metabolites with a 3 carbon atoms longer side chain. Whereas only about 1-3% of the ingested α-tocopherol appears in the urine as α-CEHC, at least 50% of ingested γ-tocopherol is excreted this way. Thus, pathways with different specificity for α- and γ-tocopherol must exist.

We investigated the degradation of α-, β-, γ- and δ-tocopherol to the corresponding CEHCs in HepG2 cells. Significantly higher amounts of γ- and δ-tocopherol were degraded to their corresponding CEHCs than of α- and β-tocopherol. A structural comparison revealed that isomers without the methyl group in the 5 position were degraded faster. 48 h after supplementation with vitamers, cells secreted significant amounts of γ-CEHC and δ-CEHC, respectively, whereas α- and β-CEHC could be detected only after long-term supplementation. Cells treated 48 h with rifampicin prior to α-tocopherol addition showed a 4-fold increase of α-CEHC secretion indicating the involvement of CYP3A4, a member of the cytochrome P450 3 family, in the first step of side chain oxidation. The data are consistent with the proposed mechanism of side chain degradation by ω- and subsequent ß-oxidation.

Endothelial dysfunction in the early stages of atherogenesis: Regulation of transcription factor activation and gene expression

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A role for LDL in endothelial dysfunction is evident, even though the molecular targets leading to alteration of endothelial homeostasis have not yet been clearly identified. Our studies demonstrate that oxidized LDL induces significant changes in the pattern of gene expression in cultured human primary endothelial cells (HUVEC) as detected by global gene expression profiling using a c-DNA array.

Transcriptional regulation is affected by ox LDL as demonstrated by an increase of both NF-κB DNA binding and transctivation activity. Using an experimental co-culture model mimicking a pro-inflammatory environment, we demonstrated that RONS generated by activated macrophages also induce NF-κB activation in HUVEC NF-κB activation in co-cultured endothelial cells was accompanied by an up-regulation of the expression of the gene encoding for the macrophage chemoattractant protein-1 (MCP-1).

Antioxidants of nutritional interest, such as α-tocopherol and catechin significantly counteracted ox-LDL dependent gene expression. Present findings strengthen the hypothesis that the early stages of atherogenesis are characterized by an altered pattern of gene and prevent experimental evidence for a protective role of nutritional antioxidants.

Session VII

Antioxidant Properties of Catechins and their Oligomers: Relationship to Health

The analysis of procyanidins in cocoa and chocolate

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It has long been known that the unfermented cocoa bean is a rich source of condensed tannis. Until recently these were analyzed by tedious purification methods followed by spectroscopy for structural elucidation. Furthermore, colorimetric techniques used for quantification were poor, as they tended to overestimate procyanidin levels. Recent hybrid HPLC/MS techniques allow for the rapid determination of the basic flavonoid structure and by employing normal-phase HPLC/MS, discrete oligomeric groupings can be identified and subsequently quantified in cocoa and chocolate based products. Further work has shown that this method may be used to analyze a broader class of proanthocyanidins in numerous fruits and vegetables. Accordingly, the normal-phased HPLC method is now being utilized in the development of a food composition database and in support of clinical feeding studies. Finally reversed-phase HPLC/MS has been used to analyze for (-)-epicatechin and its major metabolites.

Absorption and metabolism of cacao polyphenols in humans

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Cacao products are rich in polyphenols such as (-)-epicatechin (EC), catechin and procyanidins. We previously demonstrated that EC is absorbed and exists mainly as conjugated metabolites in plasma, when EC was orally administered to rat (1,2). Here we tried to evaluate the absorption of EC after intake of cacao products, chocolate and cocoa, by male volunteers. The maximum levels of total EC metabolites in plasma were reached 2 hrs after either chocolate or cocoa intake. Sulfate, glucuronide and sulfoglucuronide conjugates were the main metabolites in plasma. Urinary excretion of total EC metabolites within 24hrs was 25 ~ 30 % of total EC intake. It is therefore concluded that EC in chocolate and cocoa was partly absorbed and present as various conjugates in plasma, and then rapidly excreted in urine. We also found that the plasma after 1hr intake of chocolate was more resistant to copper ion-induced lipid peroxidation than that before intake. This result imply that intake of cacao products is helpful to improve the antioxidant defense of blood plasma.

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Absorption and metabolism of flavanols and procyanidins in the small intestine. potential *in vivo* forms and their bioactivity

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We have studied the perfusion of the jejunum and ileum in an isolated rat small intestinal model with the flavanols catechin and epicatechin and procyanidins extracted from cocoa. Perfusion of isolated jejunum with the catechin and epicatechin resulted in glucuronidation and O-methylation during transfer across the enterocytes to the serosal side. In contrast, perfusion of procyanidin oligomers resulted in no transfer to the serosal side. However, large amounts of epicatechin were detected on the serosal side after perfusion with dimer, indicating that the dimer is cleaved during transfer. Only small amounts of epicatechin metabolites/ conjugates were detected and small quantities of methylated dimer were found indicating metabolism of both monomer and dimer is restricted during dimer cleavage/translocation. Although no higher oligomers where transferred we have found that in acidic environments similar to those found in the stomach, there is decomposition of the oligomers to mixtures of monomer and dimer, thus enhancing the potential for their absorption in the small intestine.

To examine the bioactivity we have induced oxidative stress in fibroblasts using H_2O_2 and examined cellular responses in the form of mitochondrial function, cell membrane damage, annexin-V binding and caspase-3 activation. The results provide the first evidence that one of the potential *in vivo* forms of epicatechin, 3′-O-methyl epicatechin (MeEC), inhibits cell death induced by H-2O_2 and that the mechanism involves suppression of caspase-3 activity. Furthermore, the protection elicited by MeEC is not significantly different from that of epicatechin, suggesting that H-donating antioxidant activity is not the primary mechanism of protection.

Effect of Cocoa Procyanidins on Plasma Oxidation Status and Cytokine Transcription and Secretion.

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Based on numerous epidemiological studies it is thought that the consumption of flavonoid-rich diets can reduce the risk of cardiovascular disease, stroke and cancer. Potentially, this could occur as a consequence of flavonoid induced changes in plasma oxidant defense systems, or by changes in cytokine transcription and secretion. Cocoa is a flavonoid-rich food that contains substantial amounts of flavan-3-ol monomers and procyanidin oligomers. We have conducted a series of studies that examined the effects of cocoa procyanidins on cytokine transcription and secretion from human peripheral blood monocytes (PBMC). In addition, we examined the acute effects of cocoa consumption on plasma oxidation status in human subjects. In our work, we found that the larger procyanidins induced IL-1B and IL-4 gene expression and protein secretion in PHA-stimulated PBMC. Conversly, IL-2 gene expression was inhibited by the pentamer, hexamer and heptamer oligomers. In human subjects, we observed that following the acute consumption of chocolate there is a rapid rise in plasma epicatechin concentrations. Concordant with this rise, we observed a rise in plasma total antioxidant capacity, and a reduction in the plasma concentration of lipid oxidative products. Collectively, these results support the concept that flavonoid-rich foods can be associated with a number of health benefits.

Peroxynitrite and Gap junctional communication: polyphenols and selenium

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The inflammatory mediator, peroxynitrite, activates a number of signaling pathways, including MAP kinases. There are several possible mechanisms for loss of function of gap junctions, one of which is through altering the phosphorylation pattern of connexins (see recent review (1)). Short-term exposure of cells to peroxynitrite under steady-state conditions leads to a loss of cell-cell communication via gap junctions, and this can be counteracted efficiently by pretreating cells with selenite, augmenting the cellular capacity of selenoproteins, including glutathione peroxidase (2). Selenoproteins protect against peroxynitrite by direct interaction, reducing peroxynitrite to nitrite, and maintaining a catalytic activity through coupling to glutathione or other reductants.

Polyphenols have been shown to protect against peroxynitrite as well (3), so that we examined effects of polyphenols on cell-cell-communication. Interestingly, there was an increase above control levels in cell-cell- communication upon preincubation with polyphenols, such as (-)-epicatechin or genistein, which was concomitant with an inhibition of protein kinase C. However, there was a decrease upon exposure of the polyphenol-pretreated cells with peroxynitrite, *i.e.*, there was no complete protection under the conditions studied. The remaining level of cell-cell communication was still above the level of the untreated controls.

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Antioxidant and cardioprotective activity of procyanidins from *Vitis vinifera* L.

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Procyanidins from *Vitis vinifera* L. seeds are potent antioxidants. *In vitro* they act by scavenging oxygen free radicals, recycling/sparing vitamin E, stabilizing vitamin C (ESR studies), chelating transition metals and inhibiting xanthine oxidase and the respiratory burst in activated human neutrophils (1,2). This suggest that they have have a role in the prevention of I/R damage. In isolated rabbit heart Langendorff preparations, procyanidins dose-dependently reduce ventricular contracture during ischemia, improve cardiac post ischemic recovery and suppress rhythm irregularity (3). The antioxidant potential and the cardioprotective effects following ischemia/ reperfusion damage were also confirmed in *ex vivo* studies (4). Young and aged male rats were fed a diet (3 weeks) enriched with procyanidins complexed with soybean lecithin (1:3 w/w; 2.4%). At the end of the treatment, the total plasma antioxidant defense (TRAP), α-tocopherol, ascorbic acid and uric acid were determined in plasma and the hearts subjected to moderate ischema (stunning). In both young and aged rats supplemented with procyanidins, a significant cardioprotective effect was found, determined by measuring left-ventricular developed pressure, coronary perfusion pressure and the release of creatine-kinase and 6-keto-PGF1α into the perfusate. In parallel, procyanidins significantly increased the total antioxidant plasma capacity and the plasma levels of ascorbic acid while α-tocopherol was significantly lowered.

1 R. Maffei Facino et al. Planta Medica, 1998, 64, 343-347; 2 M. Carini et al Planta Medica, 2001 (in press).
3 R. Maffei Facino et al. Planta Medica, 1996, 62, 495-502; 4 R. Maffei Facino et al. Life Sciences, 1999, 64, 627-642.

Antioxidant activity and immunomodulatory action of a French maritime pine bark extract

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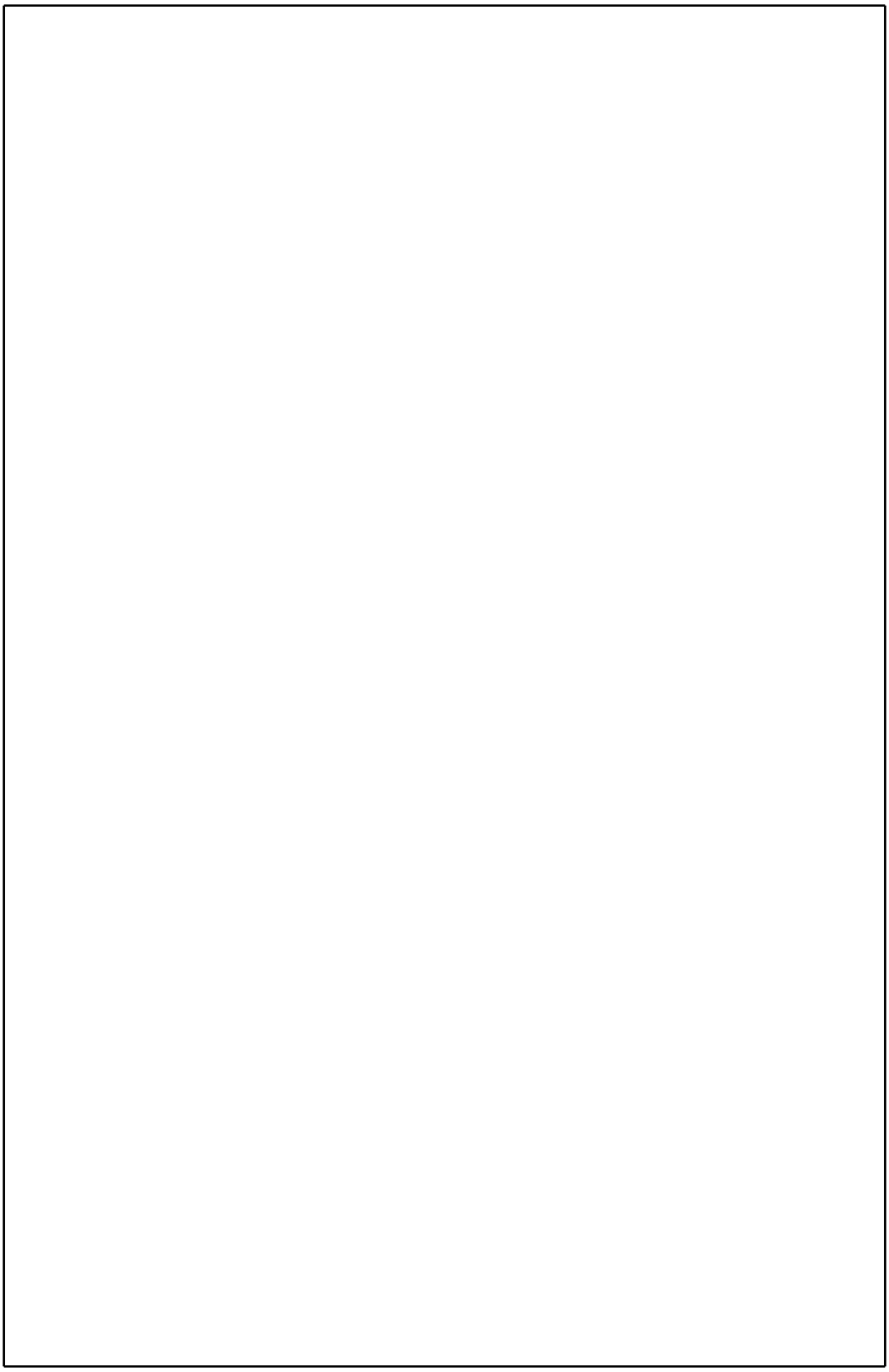
Molecular aspects of biological activity - Pycnogenol®, a Pine bark extract (PBE) from the French Maritime Pine (*Pinus maritima*) is a potent scavenger of superoxide, hydroxyl, and nitric oxide radicals. PBE intercepts radicals and spares other antioxidants such as vitamin E and glutathione within the cellular antioxidant network. Due to its high oligomeric procyanidin content (%70-75), PBE selectively binds to certain proteins. The protein binding ability together with its antioxidant properties largely accounts for the molecular basis of PBE biological activity.

Immunomodulatory action - PBE modulates inflammatory response in various cell types and exerts anti-inflammatory effect in humans. In activated macrophages, PBE shows a dual effect on NO production depending on its concentration and the characteristic of the activating signal. At low concentrations PBE increases NO production while at high concentrations it significantly inhibits NO production from LPS plus INF-γ induced macrophages. The inhibitory effect of PBE on NO production is due to the combination of several biological effects such as direct NO radical scavenging, inhibition of iNOS activity, and suppression of iNOS mRNA expression. However, PBE enhances NF-kB dependent gene expression and NO production from macrophges stimulated with IFN-γ alone, possibly due to the enhancement of TNF-α secretion. Furthermore, PBE inhibits NF-kB and AP-1 activation and thereby reduces both the production and the mRNA level of IL-1β in LPS-stimulated macrophages. In keratinocytes, PBE inhibits nuclear translocation of Stat-1 and IRF-1, important components of IFN-γ signaling pathway, and thereby suppresses IFN-γ-induced ICAM-

1 expression as well as adhesion of lymphocytes to keratinocytes. The inhibitory effect of PBE on Stat1 and IRF-1 activation indicates that in addition to the ICAM-1 gene, PBE likely also affects expression of other IFN-responsive genes that are transcriptionally regulated by Stat1 and IRF-1. Consistently, PBE treatment of keratinocytes dramatically decreases the expression of a group of genes, known to be detected in high levels in various inflammatory dermatoses such as psoriasis. Furthermore, oral supplementation of PBE significantly raises the minimal erythemal dose and prevents inflammation due to UV-light exposure of human skin. Current evidence indicates that immune system function is a major target of the modulatory action of PBE.



Session VIII
Workshop on Clinical Trials on Antioxidants: Insights and Problems

Evaluation of free radical and non-free radical scavenging antioxidant effects of polyphenols in postprandial lipoproteins

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Eating a food containing oxidized fat leads to an increase of both plasma of lipid hydroperoxides and oxidative susceptibility of LDL. Both these events are apparently related to the increase of LDL-, an oxidatively modified LDL that belongs to the small-dense subfraction. LDL- is produced by different mechanism sharing as an outcome the loss of secondary structure and conformation shift of apoB. Under different experimental conditions, the unfolding of the protein takes place due either to direct oxidative damage of specific amino acids or to perturbation of lipoprotein surface monolayer. A deeper water penetration at the interface and the shift from monolayer to micellar phase drives the protein structure derangement. In turn, a stabilization of apoB structure decreases the lipid oxidability. The biological behavior of LDL- accounts for all the observations described for *in vitro*-generated minimally modified mmLDL, of which LDL- appears as the physiological counterpart. Dietary polyphenols are known to play a protective effect in several models of atherosclerosis. Although statistically validated data on the effect of polyphenols on LDL- are not yet available, we know that procyanidines prevent the postprandial increase in both oxidative susceptibility of LDL and plasma lipid hydroperoxides. This is apparently due to an antioxidant effect during digestion. Other antioxidants that are measured in plasma, such as soybean isoflavones, may be active through a different mechanism since the free radical scavenging capacity is minimal. For those compounds, a mechanism can be envisaged similar to that of estradiol. This phenolic hormone by binding to LDL stabilizes the secondary structure of apo B and prevents the unfolding. As expected from the model, this increases oxidative stability of lipoprotein.

Glucose-6-phosphate dehydrogenase deficiency and oxidative stress in the vasculature

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The endothelial cell responds to local oxidant stress by increasing the activity of antioxidant enzymes, including glucose-6-phosphate dehydrogenase (G6PD). As a source of NADPH, G6PD serves both to modulate oxidant stress by providing the reducing equivalents necessary for glutathione reduction, and to maintain normal endothelial nitric oxide production by providing the cofactors necessary for endothelial nitric oxide synthase activity. We, therefore, hypothesized that a deficiency of G6PD perturbs these homeostatic responses. To test this hypothesis, bovine aortic endothelial cells (BAECs) were treated with the noncompetitive inhibitor dehydroepiandrosterone (DHEA) or with an antisense phosphorothioate oligodeoxynucleotide specific for G6PD mRNA (AS) to decrease G6PD activity and expression. One-hundred micromolar DHEA decreased endothelial G6PD activity by 75%, and AS also inhibited G6PD expression and activity by 76%; this degree of inhibition of G6PD was accompanied by a 38% decrease in NADPH levels. When exposed to hydrogen peroxide, G6PD-deficient endothelial cells were more susceptible to oxidant stress than were G6PD-replete cells, as monitored by cumulative changes in dichlorofluorescein fluorescence. This response was accompanied by a pronounced decrease in GSH levels owing to inefficient glutathione recycling. Cells deficient in G6PD generated less bioactive nitric oxide in response to stimulation with bradykinin or A23187, as demonstrated by a decrease in endothelial cyclic GMP production compared with G6PD-replete cells. This response could not be accounted for by a decrease in endothelial nitric oxide synthase expression or activity (measured in the presence of saturating cofactors). Preliminary studies of human volunteers defi-

cient in G6PD showed abnormal endothelium-dependent flow-mediated vasodilator responses in the forearm vasculature compared with age-matched G6PD-replete volunteers, and this blunted flow-mediated vasodilation was accompanied by an increase in F2-isoprostanes in the G6PD-deficient individuals compared with the G6PD-replete individuals. We conclude that G6PD deficiency increases the susceptibility of the endothelial cell to oxidative stress, leading to decreased nitric oxide bioavailability and endothelial dysfunction.

Antioxidants and cardiovascular disease: The need for randomized trials

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The hypothesis that antioxidant vitamins might reduce cardiovascular disease risk is based on a large body of both basic and human epidemiologic research. One of the most consistent findings in dietary research is that those who consume higher amounts of fruits and vegetables have lower rates of heart disease and stroke as well as cancer. Recent attention has focused on the antioxidant content of fruits and vegetables as a possible explanation for the apparent protective effects. Basic research provides a plausible mechanism by which antioxidants might reduce he risk of atherosclerosis. A large number of descriptive, case--control and cohort studies provide data suggesting that consumption of antioxidant vitamins is associated with reduced risks of cardiovascular disease. These data raise the question of a possible role of antioxidants, such as vitamins C and E, and beta carotene, in the primary prevention of cardiovascular disease but do not provide a definitive answer. Randomized trial data will be essential in establishing whether or not there is a causal effect of antioxidants in reducing the risk of cardiovascular disease.

For many hypotheses randomized trials are neither necessary nor desirable; however, when searching for small to moderate effects, large-scale randomized trials of adequate dose and duration, in which investigators allocate subjects at random to either active treatment or placebo will provide valuable information whether or not there is a causal relationship and provide reliable estimates of effect size. Careful consideration of issues in the design and conduct of clinical trials is essential for valid results to be achieved. Specifically, if the trial is well designed with respect to timing,

choice of study design and population, completeness of follow-up and compliance, size and duration, it can offer reliable evidence about a positive, negative or null effect of an intervention. The ultimate goal of these methodological considerations is to design studies that clearly can prove or refute the hypotheses being tested.

Results from several large-scale randomized trials of antioxidant supplements are now available, and additional trial data should be forthcoming in the near future which will better define the role of antioxidants in the primary and secondary prevention of atherosclerotic disease as well as cancer.

The Physicians' Health Study (PHS) and Women's Health Study (WHS) and Women's Antioxidant Cohort Study (WACS) are three large-scale trials that are conducted at very low cost because they are done entirely by mail. PHS is testing vitamin E, vitamin C, multivitamin and beta-carotene among older men. Women's Health Study is testing vitamin e among older women. WACS is testing vitamin E, vitamin C, beta-carotene and folate/B12/B6 in women at higher risk for CVD events.

How to develop a clinically relevant antioxidant – Alpha-lipoic acid as an example

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There is emerging evidence that oxidative stress contributes to the onset and progression of several disorders, such as diabetic complications, atherosclerosis or cancer, but there is a lack in the successful development of antioxidants. The successful development of alpha-lipoic acid may be used as an example.

Bioavailability and galenic formulation of an antioxidant.

The plasma and tissue availability is of critical importance to build up potential therapeutic levels of a potential therapeutic antioxidant. The pharmacokinetic profile of alpha-lipoic acid has been well established. The intravenous formulation is almost completely absorbed whereas the oral formulation has a first pass effect, which leads to approx. 1/3 in bioavailabil-ity when compared to the intravenous formulation. The same ratio is seen in the tissue avail-ability of the target tissue. It is of paramount importance to rec-ognize that the variability in the pharmacokinetic profile of alpha-lipoic acid also depends on the galenic formulation which subsequently influ-ences the therapeutic potential of the drug

Pharmacology of an antioxidant.

Alpha-lipoic acid's (Thioctacid®) pharmacological profile is well established. It interferes with the vascular and metabolic abnormalities of diabetic polyneuropa-thy in experimental polyneuropathy and, most importantly, in diabetic patients.

Clinical characterization of an antioxidant.

Five placebo-controlled double-blind intervention studies have been conducted to evaluate whether Thioctacid® can improve the clinically rele-vant parameters of diabetic polyneuropathy, such as symptoms, defi-cits and nerve function parameters. These studies have shown that doses of 600 mg to 1,800 mg of Thioctacid® are capable of improving these parameters.

Vitamin E atherosclerosis prevention study: Findings and perspectives

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A large body of laboratory and animal data suggest that atherosclerosis results from a series of oxidative processes. Epidemiological studies have tended to support these data by indicating an inverse relationship between vitamin E, vitamin C, carotenoid intake and cardiovascular disease (CVD) risk. But these studies are confounded by self-selection bias and early randomized controlled trials designed to determine the effects of antioxidant vitamin supplementation on cancer outcome failed to demonstrate a reduction in CVD events. Several recent randomized controlled trials designed to determine the effects of antioxidant vitamin supplementation on atherosclerosis progression and CVD events have reported mixed results in subjects either at high risk for or with established CVD. The Vitamin E Atherosclerosis Prevention Study was a randomized, double-blind, 3-year, arterial imaging clinical trial of vitamin E 400 IU/day versus placebo conducted in 353 healthy men and women $\geq$40 years of age at low risk for CVD. Initial results from this trial will be reported, compared and contrasted with recent trial results and the cumulated trial data from this important area of research summarized. More than a dozen clinical trials examining the effects of antioxidant vitamin supplementation on atherosclerosis progression and CVD events in primary and secondary prevention will be completed over the next several years. Implications of the completed and on-going clinical trials for the recommendation of antioxidant vitamin supplementation in the treatment and prevention of atherosclerosis will be discussed.

Effects of vitamin C, alone and in comparison with an antioxidant cocktail, on oxidative markers and C-reactive protein in smokers and passive smokers

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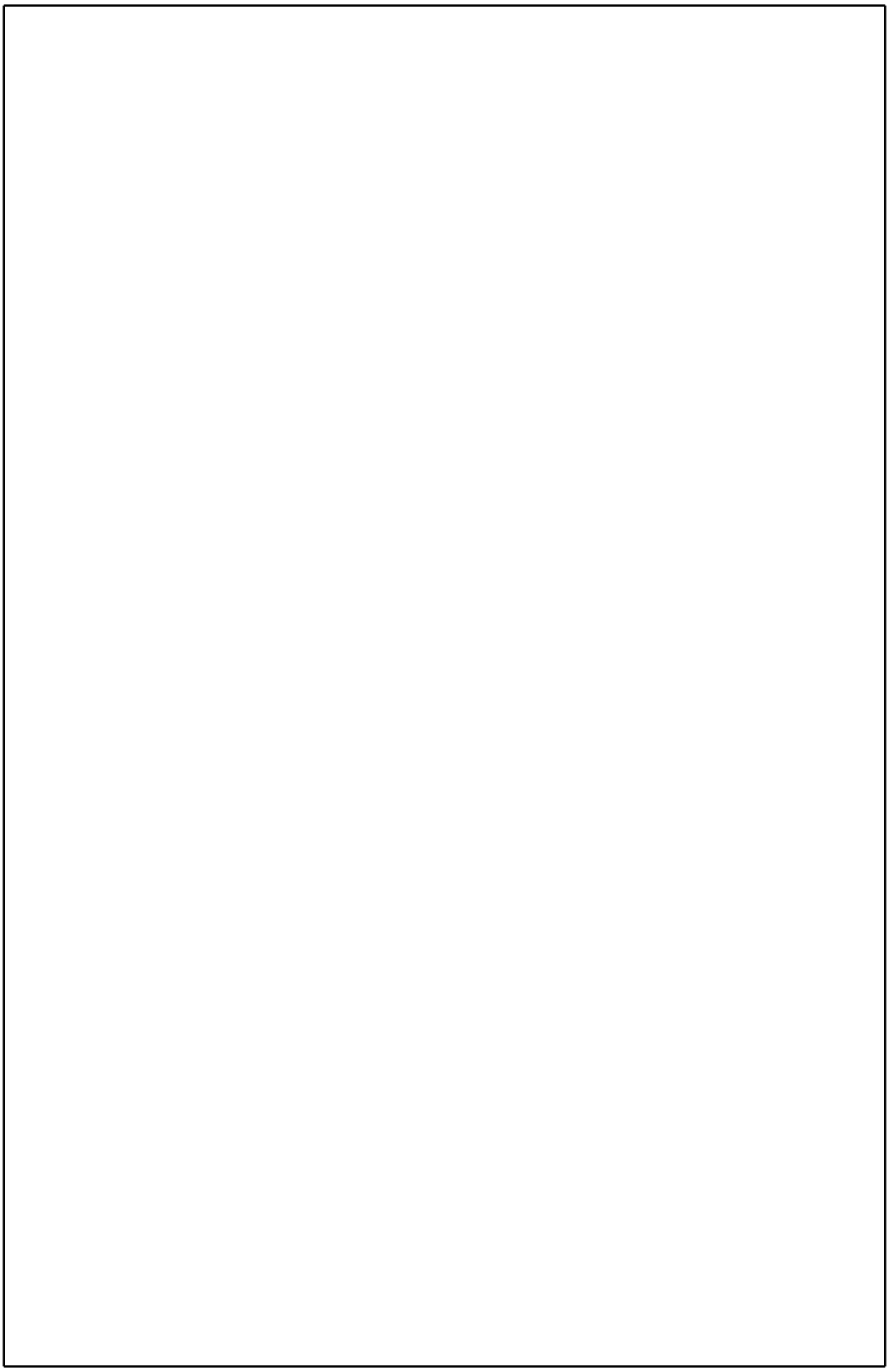
Background and Design. Smokers and passive smokers are exposed to free radicals that cause oxidative damage, contributing to the pathobiology of atherosclerosis, heart disease and cancer. It has been hypothesized that a combination of antioxidants is more effective than a single antioxidant, due to their interactions. We conducted a randomized double-blind placebo-controlled trial to investigate whether vitamin C, or an antioxidant cocktail containing vitamin C, alpha-lipoic acid, and vitamin E decrease lipid peroxidation. Two markers of lipid peroxidation were examined, plasma F2-isoprostane (IP) and malondialdehyde (MDA). Plasma of 126 smokers and 75 passive smokers (mean age 46, range 20-78 years) was analyzed at baseline and after a two-month intervention with antioxidants and placebo.

Results. Smokers with a body mass index (BMI) above the median were found to have higher IP. In that group, two months of daily supplementation with 500 mg vitamin C decreased plasma IP when compared to the Placebo group ($p=0.001$); levels in the Cocktail group were lower after treatment, but this difference was not statistically significant ($p=0.14$). Results for C-reactive protein will also be presented.

Conclusions. Vitamin C decreases smoking-related lipid peroxidation. We did not find evidence that an antioxidant combination is more effective than vitamin C alone, at least in reducing IP. The intake of vitamin C may help prevent smoking related diseases.



SESSION IX
OXIDATIVE STRESS IN NEURODEGENERATIVE DISEASES

Parkinson's disease: iron chelators as neuroprotective drugs for treatment of neurodgenerative diseases

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Neurodegenrative diseases (Alzheimer's disease, Parkinson's disease, Huntington Chorea, Amytrpophic Lateral Sclerosis, Ischaemia and Multiple Sclerosis) constitute some of the most devastating diseases known to man. They are associated with loss (death) of specific neurons in specific brain regions and when certain percentage of neuron loss occurs, the symptoms of the disease are expressed. No etiology has so far been established for many of these disorders. However, proliferation of reactive microglia and promotion of reactive oxygen species (ROS) (superoxide, hydroxyl radical and nitric oxide), with accumulation of iron at the site of neurodegeneration, is thought to participate in the process of neurodegeneration and death in all theses diseases. A pivotal role has been assigned to iron in its ability to induce oxidative stress and inflammatory cytotoxic cytokine responses resulting from redox transcription factor, NFkB, activation. In animal models of neurodegenerative diseases, employing neurotoxins (MPTP, 6-hydoxydopamine, kainite, EAE), similar deposition of iron occurs with activation of NFkB. Iron chelators and antioxidants (radical scavengers) pretreatment in animals prevent the neurodegenerative action of the neurotoxins and NFkB activation and translocation. The lessons learnt from these studies is that future treatment of neurodegenerative diseases depends on availability of effective brain penetrable iron chelatable-radical scavenging neuroprotective drugs that would prevent the progression of neuronal loss and thus the diseases. The iron chelators or radical scavengers available either do not cross the blood brain barrier or have very short half life

in vivo. We have developed a series of iron chelators which overcomes the latter. The anti-Parkinson drug, R-apomorphine, a dopamine D1-D2 receptor agonist, is an iron chelator equipotent to that of prototype iron chelator, desferrioxiamine, and crosses the blood brain barrier. In vitro, it (1μM) inhibits iron-acorbate induced membrane lipid peroxidation, and prevents hydrogen peroxide or 6-hydoxydopamine induced PC12 or neuroblastoma cell death in culture. When injected systemically it prevents MPTP and 6-hydroxydoapmine-induced nigro-striatal dopamine neuro-degeneration and Parkinsonism in mice and rats, respectively. The use of metal chelators or radical scavengers as therapeutics is not unusual when one considers the success of D-pencillamine in the treatment of Wilson's disease.

Molecular Mechanisms in Neuroprotection and Neurodegeneration in MPTP model of Parkinson's Disease: Studies from cDNA Microrrays

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cDNA microarray is a powerful method for examining the simultaneous expression of thousands of genes involved in cell pathology , drug action and drug response. We have used this approach to study dopaminergic degeneration and neuroprotection by R-apomorphine (R-APO) and the major green tea polyphenol epigallocatechin gallate (EGCG). Brain gene alterations in MPTP (N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) mice model of Parkinson's disease was investigated using Atlas mouse cDNA expression array membrane. The expression of 51 different genes involved in oxidative-stress, inflammation, glutamate and neurotrophic factors pathways as well as in still undefined processes, such as cell cycle regulators and signal transduction molecules, was differentially affected by the treatment. These findings suggest the involvement of an additional cascade of events that might act in parallel to oxidative stress and inflammation to converge eventually into a common pathway leading to neurodegeneration. The attenuation of these gene changes by R-apomorphine, an iron chelator-radical scavenger drug, supports our previous findings in-vivo where R-APO was neuroprotective. In addition, EGCG pretreatment conferred a significant protection against MPTP induced DA loss, as indicated by striatal dopamine and tyrosine hydroxylase content. The effect of EGCG effect seems to involve alterations in the expression of several signal transducers, transcriptional

repressors and growth factors mRNAs. The specific pattern of gene expression displayed by the drugs themselves, might provide hints on mechanism of action and for potential combinatory neuroprotective therapies. Conclusions: Our studies clearly indicate that the process of neurodegeneration is a cascade of events, similar to the “domino effect”. We have identified several other important events, not previously known, as a result of altered gene expression. Our results indicate that for effective neuroprotection in Parkinson’s disease and other neurodegenerative diseases a cocktail of neuroprotective drugs may be required.

Oxidative and genotoxic stress in the course of amyloid - peptide-induced neurodegeneration.

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Alzheimer's disease is characterized by the extracellular deposition in the brain of amyloid β-peptide (Aβ), presumed to play a pathogenic role. However, the molecular and cellular mechanisms involved in Aβ neurotoxicity are not yet fully understood. A dual relation exists between Aβ and reactive oxygen species (ROS). Not only can oxidative processes transform nonaggregated Aβ to toxic aggregated Aβ *in vitro*, but Aβ itself is a source of ROS. Aβ-induced neurotoxicity in human neuronal cells in culture is characterized by a rapid and transient increase of intracellular ROS and is prevented by antioxidants such as vitamin E and N-acetyl-cysteine, suggesting a role for ROS in the neurodegenerative process. Aβ in its bioactive form, the fibrillar β-sheet conformation, acts as ligand cross-linking receptors at the cell surface, triggers reactions generating cytotoxic oxidizing stimuli and thereby activates redox-regulated transcription factors and gene activation-induced cell death. To gain further insight into the molecular mechanisms underlying Aβ toxicity, differential mRNA display was used to identify Aβ-responsive genes in human preneurons NT2 at early stages of Aβ-exposure. Aβ dysregulated four main functional families of genes implicated in (i) DNA damage and repair, (ii) energetic metabolism (iii) splicing and translation (iv) cell-cell communication and cell adhesion. Of all the Aβ-dysregulated genes identified, the induction of *gadd45* (growth arrest and DNA damage inducible gene) expression is of particular interest. Gadd45 is implicated in cell cycle arrest for DNA damage repair and in maintenance of genomic stability. Nonapoptotic DNA damage, such single-strand breaks evidenced by the Comet assay, also occurs very soon after Aβ treat-

ment. DNA breakage induced by Aβ, also detected in the cortex of Alzheimer's disease victims, constitutes an early event in the pathogenic cascade leading to neuronal degeneration and is consistent with the involvement of DNA repair systems, such as *gadd45* induction.

Peroxynitrite and dopamine neurodegeneration

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MPTP causes damage to substantia nigra pars compacta (SNpc) dopaminergic (DA) neurons as seen in Parkinson's disease (PD) and to date it remains the best experimental model of PD. After systemic administration of MPTP, its active metabolite, MPP+, accumulates within SNpc DA neurons, where it inhibits ATP production and stimulates superoxide radical formation. The produced superoxide radicals react with nitric oxide (NO) to produce peroxynitrite, a highly reactive tissue-damaging species that damages proteins by oxidation and nitration. Only selected proteins appear nitrated and among these, is found tyrosine hydroxylase (TH), the rate limiting enzyme in DA synthesis and _-synuclein. The process of nitration inactivates TH and consequently DA production. Peroxynitrite also nicks DNA, which, in turn, activates poly (ADP-ribose) polymerase (PARP). PARP activation consumes ATP, and thus acutely depletes the cell energy stores. This latter event aggravates the preexisting energy failure due to MPP+-induced mitochondrial respiration blockade and precipitates cell death. Along with neuronal death, a robust glial reaction arises which contribute to enhance the neurodegenerative process. Altogether, these findings support the view that MPTP's deleterious cascade of events including mitochondrial respiration deficit, oxidative stress, and energy failure, all of which are worsened by inflammatory-related events. Because of the similarity between the MPTP mouse model and PD, it is tempting to propose that a similar scenario applies to the pathogenesis of PD and that experimental therapies targeting these different mechanisms could have neuroprotective properties in this illness.

Interaction of hydroxyalkenals with Cu,Zn-superoxide dismutase

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Germline mutations in the antioxidant enzyme Cu,Zn-superoxide dismutase (SOD1) cause a gain-of-toxic function that apparently explains 20% of the familial cases of amyotrophic lateral sclerosis (FALS). Mutant forms of SOD1 have decreased metal affinity and increased propensity to generate toxic hydroxyl radicals. It has been proposed that post-translational modification of SOD1 might cause similar alterations of activity. We now report that *in vitro* modification of wild-type SOD1 by 4-hydroxy-2-nonenal (HNE), a major product of lipid peroxidation, induces covalent dimerization of the enzyme. HNE-modified SOD has decreased affinity for Cu and Zn and increased ability to catalyze one-electron reduction of H_2O_2 to hydroxyl radical. Using an antibody against HNE-protein adducts, we have characterized hydroxyalkenal distrubution in protein fractions from the brains of Alzheimer's disease (AD)-afflicted humans. Approximately 90% of the HNE-protein immunoreactivity in the AD brain is concentrated in a single 32 kd protein. This HNE-proteins species is elevated 7-fold in the AD temporal gyrus, relative to normal brain tissue from the same region. The cerebellum, which is relatively spared in AD, contains substantially less HNE-protein adduct. Immunochemical and mass spectral evidence suggests this 32 kd protein to be a dimer of cytosolic Cu,Zn-SOD. Thus, reaction of the antioxidant enzyme SOD with products of lipid oxidation leads to metal release and formation of a potentially neurotoxic protein.

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Lysosomes as proximal messengers of cell death

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Hypothesis: Lysosomal labilization may be a common early event in apoptosis induced by a variety of agonists.

Methods: Fibroblasts, Jurkat cells, Retinal Pigment Epithelial (RPE) cells, glial and glioma cells, and three macrophage-like cell lines (J-774, U937, and THP-1) were variously exposed to: (a) chronic oxidative stress (culture under 95% O_2), (b) acute oxidative stress (H_2O_2), (c) lysosomotropic detergents, (d) lysosomotropic photo-sentitizers, (e) Cd95/FAS-activation, (f) growth factor starvation, (g) oxLDL, (h) microinjection with cathepsin D or phospholipase A2.

Results: Secondary to all these challenges, the earliest detectable event was minor to marked leakage of lysosomal contents into the cytosol (as assayed by relocation of cathepsin-D and the fluorochromic weak base acridine orange). Secondary to lysosomal rupture, but concurrent with the onset of apoptosis, cytochrome *c* relocated from mitochondria and caspase-3 was activated. Cells over-expressing Bcl-2 showed enhanced stability against the secondary, iron-independent, phase of lysosomal destabilization during oxidative stress. At least two lysosomal products may be triggering apoptosis; microinjection of cathepsin D and phospholipase A2 induced both lysosomal and mitochondrial labilization, followed by apotosis. Chelation of intra-lysosomal low-molecular weight iron, following endocytotic uptake of desferrioxamine, largely prevented oxidative stress-induced apoptosis, but not that caused by the other agonists.

Conclusion: Apoptosis following lysosomal rupture probably is due in part to direct activation of the caspase cascade by lysosomal cysteine proteases, to cytosolic release of cytochrome c following attack on mitochondrial membranes, and secondary to the activation of cytosolic lytic pro-enzymes other than pro-caspases. Lysosomal labilization is a common early event in apoptosis induced by a variety of agonists.

Retinal neurodegeneration in an in vivo model: implications for Alzheimer's disease and age related macular degeneration

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The toxicity of the so-called Alzheimer´s peptide, β-amyloid protein (AP), has been directly related to free radical damage derived from hydrogen peroxide production (Behl, et al. Cell 77, 817-27, 1994), and associated with glutathione (GSH) depletion (Müller et al., J.Neurochem. 68, 2371-7,1997) in vitro. This neuronal toxicity is prevented by vitamin E in vitro (Butterfield, Biochem. Biophys. Res. Commun. 200, 710-5, 1994). In vivo, it has been demonstrated that retinal intravitreal administration of 1-40-AP can be prevented by the coadministration of vitamin E (Jen et al., Nature 392, 140-141). Here we demonstrate the effect of intravitreal administration of the 25-35-AP on retinal GSH content, malondialdehyde (MDA), expression of bcl-2, and electro-retinogram. Wistar rats were used throughout the study. One eye of the animal was treated with aged 25-35-AP and the other was used as control or injected only with saline. Immunohistochemistry of bcl-2 and glial fibrilar acidic protein (GFAP) was performed at the end of the experiment. Electroretinograms were recorded during the 3-5 days of experiment. GSH and MDA were determined in rat whole eye homogenate (without lens) by hplc methods. Intravitreal AP induces the response of the glial cells in rat retina with an increase in the expression of bcl-2 and GFAP three days after injection. This overexpression is not accompanied by an increase in MDA concentration, though a slight decrease in GSH content (statistically significant) could be demonstrated. Electro-physiological changes were observed, specially in the b-wave of the electroretinogram, that is directly dependent on the Müller (retinal glial) cells. The effect of NMDA antagonists and other waste products on ERG are also presented. Supported by grants PM 99-0177 and FIS 99/0568 to FJR and from Fundació Bancaixa.

Role of Lutein in Prevention of Age-Related Macular Degeneration

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Lutein is one of two carotenoids which constitute the macular pigment in human retina. It has been established that lower plasma values of lutein correlate with lower levels of lutein in the eye, and that this may constitute a risk factor for developing Age Related Macular Degeneration. The mechanism by which lutein might influence ARMD is poorly understood. It would act as a blue light filter, thus preventing oxygen radical formation of the underlying photoreceptors. Since any of the major carotenoids in human plasma absorb blue light, this does not explain why lutein and zeaxanthin selectively accumulates in the human macula. It has been reported that lutein and zeaxanthin are more resistant to oxidative breakdown, than β-carotene and lycopene. Furthermore, when the water content of carotenoids in organic solutions is increased, aggregates form that no longer absorb blue light. We found however that zeaxanthin aggregates much slower than other carotenoids such as β-carotene. Properties such as resistance to free radicals and aggregation may help explain the benefit of lutein and zeaxanthin as the macular pigment. Lutein and zeaxanthin not only accumulate in the macula, but are also concentrated in the photoreceptor outer segments of the peripheral retina. We found them to be associated with extrinsic membrane proteins in the cytosol. Methods based on autofluorescence of lipofuscin have recently been developed, which can be applied to clinical and histopathological investigations of ARMD to assess the potential pathogenic role of macular pigments.

Mechanisms of Estrogen-Induced Neuroprotection

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Estrogen neuroprotective effects are multifaceted and encompass mechanisms that range from the chemical to the genomic. The data on the neuroprotective effects of estrogens indicate three levels of estrogen action, chemical, biochemical and genomic, which fall within three broad response categories, antioxidant, defense and viability[1]. A broad range of estrogens and estrogenic molecules like isoflavones and phytoestrogens can promote the chemical antioxidant effects of estrogen, a smaller subset of these chemically active estrogens can activate biochemical cascades required for neuroprotection and an even smaller subset can activate the genomic mechanisms of estrogen-induced neuroprotection[1]. Lastly, neuroprotective estrogens activate mechanisms that have the potential of protecting cells from a broad range of neurodegenerative insults that are associated with many neurodegenerative conditions including Alzheimer's disease and also can activate mechanisms specifically relevant to Alzheimer's disease. Research by our group has focused on elucidating the mechanisms underlying the full range of estrogen-induced neuroprotection. Results of these studies indicate that the neuroprotective effect of 17 beta estradiol is dependent upon activation of the MAP kinase (Erk 1 and Erk 2) and AKT kinase signaling pathways. Moreover, 17 beta estradiol-induced the anti-apoptotic protein bcl-2 expression. Estrogen-inducible bcl-2 which was blocked by the MAP kinase inhibitor, PD98059. To determine the mechanism by which 17 betas estradiol activates MAP kinase, analysis of the interaction between src, an upstream activator of MAP kinase, and estrogen was undertaken. Results of these analyses indicate that estrogen receptor α and β both co-immunoprecipitate with src suggesting a protein / pro-

tein interaction between the estrogen receptors and src. We hypothesize that this interaction occurs at the plasma membrane prior to translocation of the estrogen receptor to the nucleus where it can regulate gene transcription.

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S-ADENOSYLMETHIONINE (SAM-e) SYMPOSIUM

SAM-e: From the bench to the bedside – molecular basis of pleiotrophic molecule

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S-adenosyl-L-methionine (SAMe) was first identified by Giulio Cantoni in 1952. It was soon recognized that SAMe serves as a crucial intermediate of 3 major pathways in all biological systems: methylation, transulfuration and aminopropylation. One of the primary roles of SAMe is as the sole methyl donor in over 100 different methylation reactions. Most cells contain SAMe dependent methyltransferases that can transfer the methyl group to oxygen, nitrogen or sulfur atoms of both large and small molecules, such as DNA, proteins, phospholipids, catechol- and indole-amine s. Alternatively, SAMe serves as a precursor metabolite to the transulfuration pathway leading to the synthesis of glutathione, the major antioxidant of all cells. Furthermore, SAMe may be decarboxylated and serve as a precursor in the synthesis of the polyamines, spermidine and spermine, that are involved in regulation of cell growth. Since its discovery SAMe has been intensely studied by the scientific community in many diverse fields of biochemistry because of he pivotal role it plays in cellular metabolism. This research has provided some understanding of the mechanisms involved in the health promoting and therapeutic effects of SAMe. Stable salts of SAMe have been available for experimental pharmacological studies in animals and clinical studies in man. Clinical research studies over the last three decades have shown that SAMe has potential as a treatment for depressive disorders, liver disease and osteoarthritis. Methyl group deficiency has been implicated as a pathogenic mechanism in depression and chronic liver disease, providing a rational basis for the use of SAMe in the treatment of these disorders.

Polyenylphosphatidylcholine (PPC) opposes alcohol-induced oxidative stress and fibrosis.

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In liver diseases, already at the precirrhotic stage, there is a significant depression in the activity of phosphatidylethanolamine methyltransferase, an enzyme responsible for the production of phosphatidylcholine from phosphatidylethanolamine. Since phosphatidylcholine constitutes the backbone of all membranes, its lack results in structural and functional abnormalities of these membranes, including deficient activities of the enzymes embedded in them, such as cytochrome C oxidase, a rate limiting step in the mitochondrial electron transport chain and in energy production. Replenishment of phosphatidylcholine through administration of PPC restored the activity of cytochrome C oxidase, the mitochondrial O_2 utilization and the fatty acid oxidation. It also prevented the alcohol-induced fatty liver in rats and cirrhosis in baboons. Furthermore, PPC attenuated preexisting liver fibrosis and cirrhosis produced by CCl4 in rats. The antifibrotic effects of PPC may be due, in part, to the down-regulation of the alcohol-inducible CYP2E1 and the associated antioxidant action, documented by a normalization of markers of oxidative stress, such as F2-isoprostanes, malondialdehyde and 4-hydroxynonenal, as well as to the decrease in the number of stellate cells activated to myofibroblasts-like cells responsible for the increase in collagen production. In cultured stellate cells, PPC also stimulated the production of collagenase which opposes collagen accumulation by promoting its breakdown. Many of the beneficial effects of PPC *in vivo* can be reproduced *in vitro* either by PPC itself or by its most abundant phosphatidylcholine species, namely dilinoleoylphosphatidylcholine (DLPC), which has a high bioavailability. Because of

PPC's experimental effectiveness and its total innocuity, 18 alcoholic volunteers at a pre-cirrhotic stage were randomly given 3 daily tablets of either 1.5 gram PPC or placebo, donated by Rhone-Poulenc Rorer & Co. (Cologne, Germany). PPC significantly ameliorated markers of oxidative stress (*vide supra*). After 2 years, liver fibrosis (assessed in sequential liver biopsies), showed progression in 5 of the 9 individuals given placebo but no change or regression in the 9 subjects treated with PPC ($p<0.02$). This promising pilot study is now being verified in an ongoing much larger multi-center, double blind, randomized trial.

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S-adenosylmethionine deficiency predisposes to liver injury

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When Kinsell et al. first observed in 1947 a marked impairment of methionine metabolism in patients with liver cirrhosis, they established the crucial importance of the liver in the regulation of blood methionine concentration. A few years later, Cantoni demonstrated that the first step in methionine metabolism is its conversion to S-adenosylmethionine (AdoMet), a reaction that is catalyzed by the enzyme that is known as methionine adenosyltransferase (MAT). AdoMet is the principal biological methyl donor and the ultimate source of the propylamine moiety used in polyamine biosynthesis. In mammals, two different genes, *MAT1A* and *MAT2A*, encode for two homologous MAT catalytic subunits. *MAT1A* is expressed only in liver and it encodes for two native MAT isoenzymes, which are either a dimer (MAT III) or tetramer (MAT I) of a single α__subunit. *MAT2A* encodes for a catalytic subunit (α2) found in a native MAT isoenzyme (MAT II) which is widely distributed. *MAT2A* also predominates in the fetal liver and is progressively replaced by *MAT1A* during development. MAT isoforms differ in their kinetic and regulatory properties. MAT I and MAT II have low Km for methionine while MAT III has high Km for methionine. MAT I and MAT III are reversibly inactivated by nitric oxide and hydroxyl radicals, whereas MAT II is inhibited by physiological concentrations of AdoMet. MAT III is the truly liver-specific isoform and is thought to be responsible for clearing methionine after a protein-rich meal, whereas MAT I, like MAT II outside the liver, maintains the basal AdoMet content required by the liver

under fasting conditions.

In adult liver, increased expression of *MAT2A* is associated with rapid growth or de-differentiation of the liver. A switch in the gene expression from *MAT1A* to *MAT2A* has been observed in human liver cancer and after partial hepatectomy in the rat. Using a cell line model that differs only in the type of MAT expressed, it has been shown that cells expressing *MAT1A* exhibited the slowest rate of growth while the opposite was true for cells expressing *MAT2A.* Due to these differences in kinetic and regulatory properties, a switch in MAT expression is likely to affect the steady state AdoMet level, methylation reactions and, consequently, gene expression. Consistent with this, it was found that cells expressing *MAT1A* have much higher levels of AdoMet than cells that express *MAT2A.* Thus, the relative expression of MAT isoenzymes in liver is likely to influence the rate of liver growth and possibly facilitates the development of liver damage and hepatocarcinogenesis. We have recently generated mice lacking the *MAT1A* gene. Loss of *MAT1A* leads to increased sensitivity to develop nonalcoholic steatohepatitis (NASH) and increased liver proliferation. Since NASH is considered a progressive disease involved in many cases of cirrhosis, these results show that AdoMet deficiency plays a key role in the initiation of liver disease.

The Role of SAMe in the Treatment of Depression: A Review of the Evidence

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Major depressive disorder is very common, with a prevalence of about 17%. Although increasing numbers of people are seeking pharmacological treatment for depression, many individuals prove to be refractory to treatment, demonstrating non-response or partial response, after an adequate trial of antidepressants. In recent years there has been a surge in the popularity of natural or "alternative" medications in the US and worldwide. These medications are not as well studied as more conventional agents, and their efficacy and safety are still not clear. One of the better-studied natural agents is S-Adenosyl Methionine (SAMe), a major methyl donor in the brain, involved in the pathways for synthesis of hormones, neurotransmitters, nucleic acids, proteins, and phospholipids. There is evidence that oral or parenteral SAMe is effective for treatment of major depression. Some studies have suggested a faster onset of action for SAMe than for conventional antidepressants. SAMe may also be useful as an augmenting agent, accelerating the effect of conventional antidepressants. SAMe appears to be well tolerated, with a relatively benign side effect profile, though there are some reports of SAMe causing increased anxiety, and mania in bipolar depression. Recommended doses range from 400-1600mg/day, though some individuals may require over 3000mg/day for alleviation of depression. We will review the literature and provide recommendations on the use of SAMe for depression.

Electrophysiological neuroimaging of central effects of S-adenosyl-L-methionine (SAMe) by EEG/ERP mapping and tomography (LORETA)

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In an acute and subacute, double-blind, placebo-controlled, cross-over study, the central effects of S-adenosyl-L-methionine (ademetionine) - a naturally occurring molecule used both as a nutraceutical and as a pharmaceutical - were studied in 20 healthy volunteers of both sexes, utilizing mapping of the EEG and even-related potentials (ERP) as well as ERP tomography (low-resolution electromagnetic tomography, LORETA). 10 young subjects aged 22-33 years and 10 elderly subjects aged 56-71 years received in random order i.v. infusions of 800 mg SAMe in 250 ml of isotonic solution, and placebo administered over 30 minutes for 7 days, with a wash-out period of 3 weeks in between. EEG recordings and psychometric tests were carried out 0, 1, 3 and 6 hours, ERP recordings 0 and 1 hour after drug administration on days 1 and 7. ERPs were obtained in a two-tone oddball paradigm without motor reaction.

Multivariate analyses of the EEG results based on MANOVA/ Hotelling T2 tests demonstrated significant encephalotropic effects of SAMe as compared with placebo, which were more pronounced in the resting than in the vigilance-controlled EEG. Acute SAMe-induced changes were characterized by an increase in absolute and relative delta and theta and a decrease in alpha and beta power, further by a slowing of the delta/theta centroid and of the centroid of the total power spectrum. These pharmaco-EEG changes are typical of classical antidepressants of the thymoleptic type such as

imipramine and amitriptyline. Seven days of daily infusions increased total power, but also absolute alpha-1 and beta power. Time-efficacy calculations demonstrated a significant central effect of SAMe in the 1st hour after the first infusion as well as after 1 week of daily infusions and in the 1st and 3rd hour after one superimposed infusion on day 7 of subacute treatment.

Regarding event-related potentials, independent of subjects' age, standard N1 and P2 latencies were shortened after ADE as compared to placebo and target N2 and P300 latencies were lengthened, which reached the level of significance ($p<0.05$) for N1 and P300 latency after the superimposed treatment on day 7. N1 amplitudes increased after sub-acute treatment and temporo-occipital P300 amplitudes increased after acute dose. A trend towards P300 latency prolongation as well as an increase of the temporo-occipital P300 amplitude had already been observed in a previous study after 20 mg citalopram. As revealed by means of LORETA, N2 source strength increased in both the left and the right temporal gyrus and P300 source strength increased prefrontally, left more than right.

These EEG/ERP findings suggest both inhibitory and excitatory drug effects at the neurophysiological level, underlying the antidepressant properties of ademetionine well-documented in clinical trials. Moreover, LORETA demonstrated drug-induced increases of source strength in the (left) prefrontal cortex, a brain region that showed reduced blood flow and metabolism in depressed patients as revealed by PET.

Psychometric findings concerning attention, concentration, attention variability, numerical memory, fine motor activity, reaction time performance, well-being, somatic complaints, drive, mood, affectivity, drowsiness and critical flicker frequency showed no significant differences between SAMe and placebo, thus confirming the good tolerability of the natural compound, which is quite different from the noo- and thymopsychic changes observed after classical antidepressants such as imipramine and amitriptyline in normal volunteers.

Results of two Multicentre, controlled efficacy and safety trials of oral (MC3) and intramuscular (MC4) s-adenosyl-methionine (SAM*e*) *vs.* oral imipramine in the treatment of depression

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S-adenosyl-methionine (SAM*e*) is a natural substance which constitutes the most important methyl donor in transmethylation reactions in the central nervous system (CNS). SAM*e* proved to induce adaptive intraneuronal modifications in signal transduction mechanisms, such as an increase in cAMP-dependent microtubule-associated-protein 2 (MAP-2) phosphorylation and Ca2+/ calmodulin-dependent protein kinase activity.

A meta-analysis, carried-out on previous clinical trials, showed SAM*e* to possess significant antidepressant activity, superior to that of placebo and comparable to that of standard tricyclics. Recently intravenous (i.v.) SAM*e* (800 mg/day) has been tested in patients with major depression in two international multicentre, randomised, double-blind studies, the first *vs*. placebo (MC1) and the second *vs*. i.v. clomipramine (CLOM), 100 mg/day (MC2). In patients with severe depression (HAM-D 26) SAM*e* proved more effective than placebo (MC1), but slightly less than i.v. CLOM (MC2).

The use of i.v. SAM*e* in MC1 and MC2 limited the assessment of efficacy to hospitalised patients only, thus we decided to confirm the efficacy and safety of SAM*e* in the treatment of major depression also when administered orally (MC3) or intramuscular (MC4).

MC3 study

This 6 weeks study was carried out in 33 centres. To compare efficacy and safety, SAM*e* was given p.o., at the dose of 1600 mg/day, double-blind, *vs* oral imipramine (IMI) (150 mg/day) in patients with Major Depressive Episode (baseline HAM-D 18).

Enrolled patients were 281, 143 received SAM*e* and 138 received IMI.

According to *intention to treat analysis* (ITT) analysis of data set, the two main efficacy measures were endpoint HAM-D score and percentage of responders (final CGI score 2) at week 6. Secondary efficacy measures were considered final MADRS scores and response rate intended as a drop in HAM-D scores of at least 50% with respect to baseline. SAM*e* and IMI did not differ significantly on any efficacy measure, either main or secondary. On the other hand, adverse events were less in patients treated with SAM*e* compared to those treated with IMI.

These data show 1600 mg/day oral SAM*e* to be comparable to 150 mg/day oral IMI in terms of antidepressive efficacy, but is significantly better tolerated.

MC4 study

This 4 weeks study was carried out in 31 centres. To compare efficacy and safety, SAM*e* was given i.m., at the dose of 400 mg/day, double-blind, double-dummy *vs* oral imipramine (IMI) (150 mg/day) in patients with Major Depressive Episode (baseline HAM-D18). Enrolled patients were 295, 147 received SAM*e* and 148 received IMI.

According to ITT analysis of data set, the two main efficacy measures were endpoint HAM-D score and percentage of responders (final CGI score 2) at week 4. Secondary efficacy measures were considered final MADRS scores and response rate intended as a drop in HAM-D scores of at least 50% with respect to baseline. SAM*e* and IMI did not differ significantly on any efficacy measure, either main or secondary. On the other hand, adverse events were less in patients treated with SAM*e* compared to those treated with IMI. These data show 400 mg/day i.m. SAM*e* to be comparable to 150 mg/day oral IMI in terms of antidepressive efficacy, but is significantly better tolerated.

Due to its excellent tolerability, these data showing an equivalent efficacy *vs*. imipramine, seem to suggest interesting perspectives for the use of SAM*e*, specially for the treatment of depression in presence of any type of somatic comorbidity.

POSTERS

Free radical production in polar and temperate bivalves during heat stress

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Heat stress induces elevated metabolic performance in marine ectotherms. Basal and routine metabolism increased in the polar stenothermal bivalve *Yoldia eightsi* and in eurythermal species like *Mytilus edulis* from temperate habitats (Tremblay et al. 1998 J. Shellfish Res. 17:141) in response to heat stress. Increased MO_2 at higher temperatures is found in mitochondrial preparations of polar and temperate bivalves (see Poster A1.19) and corresponds to increased mitochondrial free radical formation (ROS) in marine ectotherms (Abele et al 2001, in review).

Rates of lipid radical formation as marker for lipid peroxidation during heat stress, increased with temperature in digestive gland of polar and temperate bivalves at comparable rates in the presence of iron. Arrhenius activation energies for lipid radical generation were 50-64 kJ/mol. Antioxidant enzyme (AOX) activities in temperate bivalves are optimized to function at maximal habitat temperatures, while under heat stress they rapidly denature and lose activity. Superoxide dismutase activities are slightly higher in polar than in temperate animals when assayed at 20° C, which may reflect compensation for activity loss at habitat temperature. Catalase in polar animals functions next to optimal also at 0° C. Heat stress-induced ROS release may be due to lower cytoplasm viscosity, and hence faster diffusion of oxygen and small metabolites to the mitochondria accelerating mitochondrial ETS activity, but may also relate to increasing mitochondrial uncoupling and higher proton leakage at higher temperatures. In addition to the decreased efficiency of the antioxidant system, oxidative stress conditions are established.

Effect of flavonoids on NADH oxidation and chemiluminescence initiated by peroxynitrite

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Peroxynitrite (ONOO-) is formed *in vivo* by a diffusion-controlled reaction (k = 1.9 x 1010 M-1 s-1) between nitric oxide and superoxide anion. Its high reduction potential indicates that it could oxidize a wide variety of biomolecules. It is of great interest to study the effects of antioxidants in preventing ONOO- damage in biological systems. The aim of the present work is to compare the ONOO- scavenging activities of several flavonoids (polyphenols and hydroxy-cinnamates). For that reason, we developed two experimental models: *a)* a *chemical system,* that involves the participation of ONOO- (200 μM), NADH (100 μM), and the flavonoids (0-200 μM) as competitive scavenger of ONOO- and *b)* a *biological system* in which the damage produced by ONOO- (200 μM) on rat liver homogenates (1-2 mg protein/ml) was evaluated in the absence and presence of plant polyphenols (0-50 μM) through chemiluminescence assay. NADH oxidation and ONOO--initiated chemiluminescence were prevented by flavonoids. When the pH was raised from 5.5 to 7.0, there was an increase of 40% and 150% in NADH oxidation and chemiluminescence, respectively. A decrease of 70% in NADH oxidation produced by ONOO- was observed following the fluorescence change in the presence of 13 mM NaHCO3 (2.5 mM CO_2 at pH 7.0). The IC50 calculated from NADH oxidation were (in μM) 275 ± 23 for (+)-catechin, 313 ± 23 for (-)-epicatechin, 144 ± 29 for caffeic acid, 173 ± 36 for chlorogenic acid and 507 ± 15 for ferulic acid. These results are in good correlation with the IC50 of the chemiluminescence assay (13 μM for (+)-catechin and 7 μM for (-)-epicatechin).
These techniques are useful to assess the ability of antioxidants to prevent ONOO- damage.

Dopamine increases the susceptibility of mitochondrial damage to NO in PC12 cells

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Parkinson's disease is characterized by a progressive loss of muscular coordination, which is due to the lower rate of production of dopamine resulting from the damage of nigrostriatal dopamine neurons. A key question that remains unanswered is why dopaminergic neurons are selectively damage in this disease. A current hypothesis refers that the presence of the dopamine molecule itself renders dopaminergic neurons more susceptible to damage. Recently, it was found that NO production increases during the progress of Parkinson disease as a result of inflammation-like processes and has key role damaging dopaminergic neurons. Because NO catalyses the oxidation of dopamine, a logical hypothesis is that dopamine renders dopaminergic cells more susceptible to the damaging effects of NO. We decided to explore the interaction between NO and dopamine in the context of mitochondrial damage, because the mitochondrion is a preferred target for the damaging effects of NO and its dysfunction is probably a key event in the damage of dopaminergic neurons.

In PC12 cells, we found that dopamine by itself does not damage complex I-driven respiration, but strongly potentiates the damage exerted by NO. Conversely, depletion of dopamine from PC12 cells had a protective effect against NO damage. We conclude that in PC12 cells NO and dopamine act synergistically damaging mitochondrial respiration.

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Antioxidant cellular responses: potential role of ergothionene in cellular homeostasis

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Diet-derived and drug-derived antioxidants may be particularly important in protecting against a number of human diseases, provided the active components become bio-available and are seen to have defined physiological functions e.g. up-regulation of defense antioxidants, modulation gene expression (with respect to synthesis of DNA repair enzymes), effect on cellular transduction mechanisms, reduction of *in vivo* markers of oxidative stress and/or reduction of disease risks. Ergothioneine (2-mercaptohistidine trimethylbetaine) (EGT)) is a naturally occurring antioxidant that is abundant in most plants and animals. The compound is formed via hercynine from histidine, methionine and cysteine in microorganisms and is not bio-synthesized in animals. As such it can only be derived from plant diet. Human blood values have been reported as 4-17mM. Ergothioneine is not toxic to different cell lines with IC50s up to 50 mM. Supplementation of cells with increasing concentrations of ergothioneine permits the cells to tolerate high concentrations of N-acetylcysteine. Ergothioneine is suggested as a unique antioxidant buffer, which has the capacity to provide and maintain cellular homeostasis. Ergothioneine reduces the oxidant dependent release of proinflammatory cytokine IL-8. In the context of pulmonary inflammation disease, ergothioneine may become an important OTC antioxidant prophylactic agent depending on formulation.

Peroxynitrite induces mitogen-activated protein kinase phosphorylation *via* protein kinase C

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Peroxynitrite (ONOO-) exposure results in phosphorylation of mitogen-activated protein kinases (MAPKs). In the present study the mechanism of extracellular signal-regulated kinase (ERK1/2) phosphorylation by ONOO- has been investigated in rat-1 fibroblasts. Incubation of the cells with ONOO- resulted in a dose and time-dependent phosphorylation of ERK. ONOO- mediated ERK phosphorylation could not be blocked with PD98059, an inhibitor of MAPK kinase (MEK). Further, we did not observe an increase in MEK phosphorylation indicating that ERK activation by ONOO- occurred independent of MEK. To investigate whether protein kinase C (PKC) might be involved, we utilized PKC down-regulator phorbol-12, 13-dibutyrate (PDBU). Pre-incubation of cells with PDBU for 24h to down regulate PKC, resulted in a significant reduction of ONOO- induced ERK phosphorylation. This implies that activation of MAPK by ONOO- depends on PKC activation. Indeed, PKCα and ε were activated upon ONOO- exposure. Moreover, when cells were treated with ONOO- in a calcium-free buffer, PKCα was not activated. Interestingly, a marked inhibition of ERK phosphorylation was also observed suggesting the involvement of calcium in the effect of ONOO-. In conclusion, the data demonstrates that phosphorylation of ERK1/2 following ONOO- exposure occurs *via* a calcium and PKC-dependent but MEK-independent pathway.

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Effects of targeted intramitochondrial expression of catalase in transgenic *Drosophila melanogaster*

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Ectopic expression of catalase in the mitochondria of *Drosophila melanogaster* has been accomplished using a transgenic approach, in which an ornithine aminotransferase (OAT) leader sequence was fused in frame to the coding sequence of the genomic catalase gene. The resulting transgenic flies were previously shown to have dramatically (~90%) decreased rates of mitochondrial hydrogen peroxide release. The goal of the current study was to examine the effect of this artificial expression of catalase on the aging of the flie. The main hypothesis was that ectopic expression of catalase and the resulting decrease in H_2O_2 release from mitochondria would extend the life span of the organism. Total catalase activity was increased by an average of 46% at 10 days of age and 85% at 53 days of age, in a comparison of 10 experimental and 10 control lines. Mitochondrial catalase activity in the experimental lines ranged from 31 to 139 U/mg protein versus 0 to 9 U/mg protein for the controls. Preliminary life span experiments suggest a marginally significant decrease (6.3%) in the mean longevity of the OAT-catalase expressors compared to the control lines. Hyperoxia experiments (100% oxygen exposure) on these same lines revealed an average increase of 4.2% in the survival time of experimental versus control flies. These results are consistent with previous findings indicating that single antioxidant gene overexpression has little or no effect on the life span of *Drosophila*. Future experiments will determine whether mitochondrial expression of catalase is also neutral or deleterious at the level of protein, lipid and DNA oxidative damage.

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Degradation of oxidatively-denatured aconitase by the *lon* protease in mitochondria

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Our previous results have shown that mitochondria contain a proteolytic system able to recognize and degrade oxidatively-denatured proteins. We have continued the characterization of this system in bovine heart mitochondria, using aconitase as a substrate. The proteolytic susceptibility of aconitase gradually increased after *in vitro* treatment with H_2O_2 up to a concentration of 5mM. At this oxidant exposure the rate of aconitase degradation was 7 times higher than that of unoxidized aconitase. When subjected to higher concentrations of H_2O_2 aconitase proteolysis decreased, returning to basal levels at 20 mM. Further studies of the proteolytic activity responsible for selective degradation of oxidized aconitase suggested the involvement of an ATP-stimulated serine protease. For example, the degradation of both oxidized and unoxidized aconitase was strongly inhibited by PMSF and partially inhibited by NEM. Proteolysis could be stimulated by ATP addition (in the presence of 10 mM ATP the activity was increased 4-6 fold) but the rate of proteolysis was increased by the same percentage for both oxidized and control substrates. EDTA also slightly decreased the proteolytic activity in the absence of ATP, but almost completely blocked the ATP-stimulated portion of protein degradation. Non-hydrolysable ATP-analogs could not stimulate the degradation of oxidized aconitase, suggesting that the energy of ATP hydrolysis is used to stimulate proteolysis of oxidized aconitase. All our data matched the inhibitor/activator profile of the Lon protease, so we decided to purify this enzyme from mitochondria and study its capacity for degrading oxidized aconitase in vitro. Our results indicate that the purified mitochondrial Lon protease can indeed preferentially degrade oxidized aconitase in an ATP-stimulated manner.

Increase of glutathione and glutathione S-transferase levels after gamma irradiation of Sertoli cells and astrocytes in culture

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The aim of this study was to determine the ability of two radioresistant normal cells, Sertoli cells and astrocytes, to respond to a radio-induced oxidative stress. Glutathione (GSH) and glutathione S-transferases (GSTs) are known to play a key-role in the anti-oxidative defense by scavenging reactive oxygen species (ROS), conjugating ROS electrophiles by-products and eliminating DNA peroxides through their peroxidase activity. Sertoli cells and astrocytes were isolated from rat testis and brain respectively and established in primo-culture. Irradiation was performed using a ^{60}Co unit (3 to 21 Gy). The total intracellular GSH and total GSTs activities were determined spectrophometrically at different times after irradiation. In these conditions, we observed an increase in the total GSH content in Sertoli cells and astrocytes in a time and dose dependant manner. In Sertoli cells irradiated at 21 Gy, a 2 fold increase in GSH levels was observed. GSTs activity increases in the same range for Sertoli cells but doesn't change for astrocytes. We conclude that these two radioresistant cell types have the ability to respond to a radio-induced oxidative stress by increasing their defense systems (GSH and GSTs). The differences between Sertoli cells and astrocytes response could be due to the fact that the basal level of GSH and GSTs activities is much more important in astrocytes compared with Sertoli cells.

Effect of chronic enalapril and losartan treatments on mitochondria function in rat kidney

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Oxidative damage to mitochondria (MT) could account for some of the age-related changes of MT functions. We studied whether chronic enalapril (E) or losartan (L) treatments affect MT functions. MaleWistar rats (14-mo old) were divided in 3 groups and administered with E (10 mg/kg/d), L (30 mg/kg/d), or water (C) for 8 mo. Kidney homogenates, supernatants, and MT fractions were prepared by differential centrifugation. MT O_2 consumption was measured in a two channel oxygraph. H_2O_2 production, enzymes activities, and GSH were determined spectrophotometrically. After 8 mo on the respective treatment, body and organ weights were similar among the groups. The MT respiratory control ratio (state 3:state 4) was higher in E and L that in C (E = 6.1±1.3*; L = 5.4±1.1*; C = 3.0±0.7; *$p<0.05$). H_2O_2 production (nmol/min/mg protein) was lower in the E and L groups than in C (E = 7.2±1.2*; L = 7.1±1.3*; C = 10.0±0.1; *$p<0.05$). Kidney total glutathione content was higher in L than in C (1.25±0.12* vs 0.81±0.11 µmol GSH/mg protein); and kidney GSH/GSSG was higher in both E and L than in C (E = 7.2±2.2*; L = 9.6±3.9*; C = 3.1±1.4, *$p<0.05$). These results indicate that the inhibition of the renin-angiotensin system could act regulating kidney MT respiration, and ameliorating certain functional changes that occur with age.

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JNK Activation by Hydrogen Peroxide in Endothelial Cells Involves Src-dependent EGF Receptor Transactivation

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The phenotypic properties of the endothelium are subject to modulation by oxidative stress and the c-Jun N-terminal kinase (JNK) pathway is important in mediating cellular responses to stress, although activation of this pathway in endothelial cells has not been fully characterized. Therefore, we exposed endothelial cells to hydrogen peroxide (H_2O_2) and observed rapid activation of JNK within 15 min that involved phosphorylation of JNK and c-Jun, and induction of AP-1 DNA binding activity. Inhibition of protein kinase C and phosphoinositide 3-kinase did not effect JNK activation. In contrast, the tyrosine kinase inhibitors, genistein, herbimycin A, and PP2 significantly attenuated H_2O_2-induced JNK activation as did endothelial cell adenoviral transfection with a dominant-negative form of Src, implicating Src as an upstream activator of JNK. Activation of JNK by H_2O_2 was also inhibited by AG1478 and antisense oligonucleotides directed against the epidermal growth factor receptor (EGFR), implicating the EGFR in this process. Consistent with this observation, H_2O_2-induced EGFR tyrosine phosphorylation and complex formation with Shc-Grb2 that was abolished by PP2 implicating Src in H_2O_2-induced EGFR activation. Tyrosine phosphorylation of the EGFR by H_2O_2 did not involve receptor autophosphorylation at Tyr 1173 as assessed by an autophosphorylation-specific antibody. These data indicate that H_2O_2-induced JNK activation in endothelial cells involves the EGFR through a Src-dependent pathway that is distinct from EGFR ligand activation. These data represent one potential pathway for mediating oxidative stress-induced phenotypic changes in the endothelium.

Substance P through neurokinin 1 receptor facilitating afferent pathway and promoting free radicals release in rat bladder

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We studied the effect of substance P (SP), either directly or indirectly via the reactive oxygen species (ROS) formation, in the hyperactivity of bladder associated with neurogenic inflammation. We used cystometrogram, pelvic afferent (PANA) and efferent nervous activity (PENA) to characterize the mechanism underlying SP-induced bladder hyperactivity. *In vivo* and *in vitro* measurement of ROS generation and localization was determined by chemiluminescence (CL) and dichlorofluorescin (DCFH) diacetate fluorescence staining techniques. In response to saline filling to a threshold volume/pressure, the bladder revealed a PENA-mediated micturition reflex triggered by enhanced mechanoreceptor-dependent PANA. Results showed that this reflex can be partially inhibited by intra-arterial (i.a.) or intrathecal (i.t.) CP96,345, a neurokinin 1 (NK1) receptor antagonist, but not SR48968, a NK2 receptor antagonist. Exogenous SP can evoke early-myogenic contraction, excite PANA to facilitate bursting PENA mediated contractions. The exogenous SP-effect can be partially prevented by i.a. or i.t. CP96,345. Our study further revealed that exogenous SP caused increase in ROS levels in bladder and whole blood via mast cell degranulation and neutrophil activation. The neutrophils were the primary source of SP-enhanced ROS. Treatment with CP96,345 or free-radical scavengers reduced the ROS amounts in bladder and whole blood, and ameliorated the hyperactive bladder response. We conclude that SP induced NK1 receptor activation in an upstream afferent pathway, promoting downstream ROS release, and subsequently developing a neurogenic bladder. NK1 receptor blockade or free-radical scavengers may be a therapeutic application to ameliorate hyperactive bladder.

Apheresis with Ascorbic Acid Treatment Prevents Hydrogen Peroxide Induced Oxidative Stress

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Oxidative stress, especially phosphatidylcholine hydorperoxide (PCOOH), is elevated in the hyperlipidemic or uremic patients after apheresis and may play a role in the pathogenesis of atherosclerosis. We evaluated the effect of low-density lipoprotein (LDL) apheresis with ascorbic acid treatment on plasma H_2O_2 and HOCl activity, LDL and PCOOH response by emission spectrometric and high performance liquid chromatographic chemiluminescence in uremic or hyperlipidemic patients. We found that (1) LDL apheresis augmented plasma H_2O_2 and HOCl activity and decreased total antioxidant status (TAS) in these patients; (2) ascorbic acid administration increased TAS and selectively diminished LDL-apheresis induced plasma H_2O_2 activity, but not HOCl activity; (3) the degree of lipid oxidation [PCOOH and thiobarbituric acid reactive substance (TBARS)] and protein oxidation in uremic or hyperlipidiemic plasma was significantly reduced by apheresis; and (4) reduction of LDL levels by apheresis was similarly reduced PCOOH, but not TBARS. We conclude that LDL apheresis with ascorbic acid administration can efficiently increase antioxidant defense and reduce oxidized LDL induced oxidative stress.

Further evidence for altered processing of oxidative DNA damage in systemic lupus erythematosus

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Given the significant inflammatory component in autoimmune disease, systemic lupus erythematosus (SLE), a condition of oxidative stress, would be anticipated in this disease. Experimental evidence for this derives from numerous studies reporting elevated markers of oxidative damage to DNA and lipids. Lowered serum antioxidant capacity and a propensity to develop autoimmune disease further links oxidative stress and SLE. There appears to be growing evidence that the repair of oxidative DNA damage, such as 8-oxo-2'-deoxyguanosine (8-oxodG), in SLE is impaired. Indeed, we have recently reported evidence for the attenuated repair of 8-oxodG in SLE, which coincides with autoimmune phenomena in several DNA repair- deficient conditions.

We previously reported on an ELISA method which has been used to identify the presence of single-stranded DNA oligomers in urine, with levels significantly elevated in SLE and psoriasis. Further analysis has shown that, in control subjects, urinary oligomers are positively correlated with urinary 8-oxodG ($r = 0.54$). In contrast, a negative correlation between urinary oligomers and 8-oxodG were noted in SLE ($r = -0.66$). Surprisingly, levels of urinary thymine dimers, a product of UV irradiation, correlated positively with oligomers, in both SLE and control subjects ($r = 0.61$ and 0.82, respectively). Preliminary analysis of control data is supportive of our hypothesis that at least a proportion of the oligomers derive from nucleotide excision repair (NER), with lesions present within such fragments. The SLE data revealed a radically reversed trend, with high urinary 8-oxodG corresponding to low oligomer levels. These data imply a profound alteration in SLE of a pathway responsible for the removal of 8-oxodG, possibly NER. Studies are currently underway to develop a more refined immunoassay and investigate this phenomenon further.

Effect of dopamine and NO· on complex I-driven respiration and ANT activity in mitochondria

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Oxidative stress is believed to cause mitochondrial alterations occurring in dopaminergic cells in substantia nigra associated with Parkinson's disease. It is hypothesized that increased generation of NO· in response to the activation of the inducible form of nitric oxide synthase in glial cells accelerates the autoxidation of dopamine leading to enhanced production of O_2·-, ONOO- and dopamine quinones. This pathway contributes to increase the oxidative load of the dopaminergic cells and consequentially the oxidative/ nitrosative damage to mitochondria.

In this study, we evaluated the effect of dopamine and NO· on complex I respiration and the inibition by NO· of ANT (adenine nucleotide translocase), a *sensor* of oxidative stress.

We demonstrated that NO· plays a crucial role in the dopamine autoxidation and mitochondrial oxidant production. These results provide evidence for an inhibition of mitochondrial complex I activity during the metabolism of NO·, involving both ONOO- and dopamine quinone formation.

Two mitochondrial targets that we propose to be selectively damage by the system NO·/dopamine are complex I and ANT.

Comparison of plasma antioxidant levels of passive smokers, smokers and nonsmokers, with adjustment for dietary antioxidant intake

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Background: Free radicals in cigarette smoke (CS) cause oxidative damage to macromolecules, contributing to the pathobiology of atherosclerosis, heart disease and cancer. *In vitro* studies have shown that antioxidants (AO) quench free radicals. *In vivo*, low levels of plasma antioxidants may indicate elevated oxidative stress status. Objective: In this study we compare plasma AO levels, and the ratio of dehydroascorbic acid (DHAA) to total ascorbic acid (TAA), of passive (PS) and active smokers (S) with nonsmokers (NS) to investigate whether not only active smoking but also passive smoking is an oxidative stress factor. Design: Plasma samples of 83 S, 40 PS and 36 NS were analyzed for ascorbic acid, α- & γ-tocopherol, 5 major carotenoids, retinol and cotinine. Subject groups were compared by one-way ANOVA with adjustment for race, sex, age, BMI, alcohol, triglycerides, fruit and vegetable intake, and dietary AO. Results: S and PS have significantly lower levels of plasma β-*carotene* than NS (S vs NS: p=0.06, PS vs NS: p=0.01). S have lower plasma levels of *vitamin C and* β-*cryptoxanthin* than PS and NS (vit.C: S vs PS p=0.01, S vs NS: p=0.02; β-crypt: S vs PS p=0.02, S vs NS p=0.03). S have lower levels of *lutein and zeaxanthin* than NS (p=0.01). S and PS have significantly higher plasma levels of γ-*tocopherol* (p=0.02 in both comparisons; unadjusted for dietary γ-tocopherol). No significant differences in plasma levels of the other AO were observed. Since we controlled for dietary intake of AO, the results do not reflect differences in dietary habits of the subject groups. Results on the ratio of DHAA to TAA in plasma of S, PS and NS will be presented at the meeting. Conclusions: These results indicate that active and passive smoking are factors that are associated with increased oxidative stress.

Overexpression of the calcineurin inhibitory gene *DSCR1 (Adapt78)* is associated with Alzheimer's disease

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The *DSCR1 (Adapt78)* gene was independently discovered as a resident of the "Down Syndrome Candidate Region," and as an "Adaptive Response" shock or stress gene that is transiently induced during oxidative stress. Recently the *DSCR1 (Adapt78)* gene product was discovered to be an inhibitor of the serine/threonine phosphatase, calcineurin and its signaling pathways. We found significant expression of *DSCR1 (Adapt78)* in brain, spinal cord, kidney, liver, mammary gland, skeletal muscle, and heart. In the brain *DSCR1 (Adapt78)* is predominantly expressed in neurons within the cerebral cortex, hippocampus, substantia nigra, thalamus, and medulla oblongata. Based on all these data we hypothesized that *DSCR1 (Adapt78)* might be involved in the development of Alzheimer's disease. To address this question we compared *DSCR1 (Adapt78)* mRNA expression in *post mortem* brain samples from Alzheimer's disease patients and individuals who had died with no Alzheimer's diagnosis. *DSCR1 (Adapt78)* mRNA levels were found to be about twice as high in age-matched Alzheimer's patients as in controls. *DSCR1 (Adapt78)* mRNA levels were actually three times higher in patients with extensive neurofibrillary tangles (a hallmark of Alzheimer's disease) than in controls. There was no correlation between patient age and *DSCR1 (Adapt78)* mRNA levels. Using a cell culture model we found that the amyloid a$1-42 peptide, which is a major component of senile plaques in Alzheimer's, can directly induce increased expression of *DSCR1 (Adapt78).* Our findings associate *DSCR1 (Adapt78)* with such major hallmarks of Alzheimer's disease as amyloid protein, senile

plaques, and neurofibrillary tangles. a$ may chronically induce *DSCR1 (Adapt78)* which inhibits the serine/threonine phosphatase activity of calcineurin, causes tau hyperphosphorylation and formation of neurofibrillary tangles, promoting Alzheimer's disease.

Vitamin E metabolism in systemic lupus erythematosus

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The autoimmune disease systemic lupus erythematosus (SLE) is characterised by autoantibodies recognising multiple nucleic acid/ nucleoprotein antigens, including double stranded DNA. The pathogenesis of SLE is complex with a potential role for oxidative stress; elevated markers of oxidative damage to lipid and nucleic acid are seen along with lowered plasma antioxidant status. We have reported changes in the level of the oxidative DNA lesion 8-oxo-2'-deoxyguanosine (8-oxodG) in peripheral blood cells, serum and urine of patients with SLE recruited to a placebo-controlled vitamin C supplementation study1. This study showed attenuated 8-oxodG excretion during vitamin C supplementation compared to healthy subjects, and we attributed this to an impaired ability to process this lesion in SLE.

Selected urine samples from the SLE patients participating in the oral vitamin C supplementation study (500mg/day) were analysed for vitamin E metabolites [α-tocopheronolactone (α-SM); carboxyethylhydroxychroman metabolites (α-CEHC, γ-CEHC)] by gas chromatography-mass spectrometry using isotopically labelled internal standards. In SLE, levels of α-CEHC and γ-CEHC were significantly ($p<0.05$) elevated compared to healthy subjects, whilst plasma vitamin C showed a significant negative correlation with α-SM ($r=-0.56$, $p<0.05$). No significant correlations were observed with these metabolites for the healthy subjects.

The α- and γ-CEHC metabolites, resulting from phytyl side chain

oxidation, may be derived from the metabolism of excess tocopherols by cytochrome P-450 activities. On this basis, the data suggest that SLE patients are either replete with vitamin E, or catabolism of tocopherols is elevated. For the α-SM metabolite, possibly a product of oxidative turnover of α-tocopherol, significant negative correlation with plasma vitamin C implies a protective effect of vitamin C on α-tocopherol. The latter is not unreasonable given a proposed role for vitamin C in the regeneration of oxidised tocopherol in plasma membranes. Vitamin E may have multiple protective roles in a chronic inflammatory disease such as SLE where there is evidence of increased oxidative stress. Recent studies in a murine model of SLE have indicated the possibility that vitamin E can ameliorate disease progression perhaps suggesting a viable adjunct to traditional therapies.

1. M.D. Evans *et al.* (2000) Biochem. Biophys. Res Comms **273**: 894-898.

Modification of proteins and lipids by hypochlorite

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The reactive oxygen species HOCl, which is produced by myeloperoxidase, can modify proteins and lipids by chlorination and/or oxidation reactions. In proteins there appears to be a sequential order of modification with the thiol group of cysteine as the most susceptible side chain. We explored the consequences of this sequential order for the activity of a model membrane enzyme, sarcoplasmic Ca-ATPase, which is essential for calcium homeostasis in skeletal muscle. In contrast to published data already low-micromolar concentrations of HOCl led to a significant loss of activity by a process with two distinctly different kinetics. During the first stage there was hardly any loss of activity irrespective of the HOCl concentration while in the subsequent stage the loss of activity was dependent on the HOCL concentration and only weakly delayed by the HOCl scavenger taurine. These results support the recently proposed concept of non-essential Cys residues as "endogenous" antioxidants. Furthermore the generally accepted strategy for the determination of IC50 values by measuring initial velocities is challenged since our continuous method for the monitoring of the effect of HOCl showed that initial velocities did not reveal the full inhibitory potential of the oxidant. For the modification of lipids both peroxidation and chlorohydrin formation have been described for unsaturated fatty acids. We studied by LC-MS which of these reactions predominates in phosphatidylcholines (PC) of low density lipoprotein (LDL) when LDL was treated either with hypochlorite or the MPO/H_2O_2/Cl-system. Several PC chlorohydrins were detected, but no PC hydroperoxides as primary products of lipid peroxidation could be observed.

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Inhibition of neutrophil apoptosis by acrolein: a contributing factor in tobacco-related inflammatory lung disease?

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Cigarette smoking is known to contribute to inflammatory diseases of the respiratory tract by promoting recruitment of inflammatory-immune cells such as neutrophils and perhaps by altering neutrophil functional properties. We investigated whether acrolein, a toxic unsaturated aldehyde found in cigarette smoke, could directly affect neutrophil function. Exposure of freshly isolated human neutrophils to acrolein was found to markedly inhibit spontaneous neutrophil apoptosis, as indicated by loss of membrane asymmetry and DNA fragmentation. In addition, acrolein induced neutrophil production of the chemokine interleukin-8 (IL-8). Studies of signaling pathways revealed that acrolein (1-50 μM) induced a marked activation of ERK and p38 mitogen-activated protein kinases (MAPKs), and acrolein-induced IL-8 release was prevented in the presence of the p38 MAPK inhibitor SB203580. However, inhibition of either ERK or p38 MAPK did not affect acrolein-dependent inhibition of apoptosis. Acrolein exposure prevented the activation of caspase-3, a crucial step in the execution of neutrophil apoptosis, presumably by direct inhibition of the enzyme. Our results indicate that acrolein may contribute to smoke-induced inflammatory processes in the lung, by increasing neutrophil recruitment and reducing neutrophil clearance by apoptosis.

Can DZQ Target Cells with a High Dt-Diaphorase Activity?

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The effectiveness of many chemotherapeutic agents may be compromised due to many limiting factors of specificity and dependence on p53, a tumor suppressor gene. Many chemotherapeutic strategies are targeted at inducing p53 levels in cancer cells thereby eliciting cell cycle arrest and/or apoptosis. Unfortunately, over 50% of the cancer cells are p53 deficient, hence, the importance of developing new targets for anticancer therapy. Aziridinylbenzoquinones are a group of anticancer agents with both alkylating and redox cycling properties. We have already shown the efficient metabolism of 3,6-diazirid inyl-1,4-benzoquinone (DZQ) and induction of p21 by cells with a high DT-diaphorase (NAD(P)H:quinone oxidoreductase) activity and minimal metabolism and induction of p21 by cells with low activity. A high DT-diaphorase activity is a unique characteristic inherent in only some cancers, while wild type cells posses a low activity. Cell proliferation was inhibited while aconitase activity exhibited an initial activation and subsequent decrease, which suggests that DZQ may posses the ability to target and elicit its effects specifically to cells, endowed with a high DT-diaphorase activity.

Activation of antioxidant and neuronal genes by a herbal extract Kishorchandra

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Extracts from plants are used for the prevention and treatment of human diseases but their mechanisms of actions are poorly defined. Application of recently developed tools for the analysis of signal transduction pathways and global gene expression have identified molecular targets affected by plant derived antioxidants in mammalian cells. We have recently shown that an extract from leaves of Ginkgo biloba (EGb 761) targets the transcription of selected genes in a human cancer cell. The most remarkable feature of this study was the activation of ~150 genes. One of these genes coded for γ-glutamyl cysteinyl synthetase (γ-GCS), the rate controlling enzyme in the synthesis of glutathione. We have now screened additional cell types and demonstrated that this is an ubiquitous effect of the extract on the transcription of γ-GCS. In vivo studies with mice whose diets were supplemented with EGb 761 for 4 weeks also showed activation of transcription of several genes in the brain. The activated genes encode proteins important in memory, learning and neuroprotection. This shows that the extract has neuroactive compounds and supports numerous pharmacological, biochemical, and behavioral studies. More importantly, the study identifies the genes that may contribute to the neurological effects of Ginkgo biloba extract in vivo. In summary analysis of global gene expression suggests that EGb 761, a herbal extract, activates transcription of selected genes in vitro and in vivo.

A cell model for production of biologically important metabolites of wine flavonoids

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Red wine consumption has beneficial effects on cardiovascular health. A recent study has shown that wine consumption can lower the mortality risks from cardiovascular disease and cancer more significantly than can be attributed solely to alcohol intake. De-alcoholized red wine can reduce formation of aortic lipid deposits in a hyperlipidemic hamster model as efficiently as intact red wine. Those studies show clearly that red wine solids contain compounds that have beneficial effects on cardiovascular health. Determination of catechin in blood of humans after ingestion of a single serving of red wine revealed nearly undetectable levels of the parent compound. The vast majority of the ingested flavonoid was found in methylated, glucuronidated and sulfated form. This raises the question whether it is the parent flavonoid or the derived metabolites that are the active compounds. Further investigations of flavonoid metabolites have not been possible due to the lack of availability of these metabolites.

We have successfully established a cell culture model that produces physiologically important metabolites of individual flavonoids. Purified metabolites were analyzed by high-performance liquid chromatography coupled with mass spectrometry. We verified that the metabolites produced with the cell line (methylated/ glucuronidated/sulfated) were the same as those found in humans after ingestion of the parent flavonoid.

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Reactive oxygen species and vitamin E in the aquacultured sea bass testis

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Reactive oxygen species and lipid peroxidase are normally produced by the testis. Protection against reactive oxygen species is provided by antioxidants that limit peroxidation, a factor compromising the normal development of sperm and the capacity of fertilization. Our objectives were to establish the testis levels of the antioxidant vitamin E during the different phases of gonadal development and determine a relation with plasma vitamin E and plasma sex hormones fluctuations in the highly prized aquacultured sea bass, *Dicentrarchus labrax*. Vitamin E was assayed using HPLC and the sex hormones by RIA.

Testis and plasma vitamin E concentrations paralleled the androgens levels and increase during the gonadal recrudescence, whereas both significantly decreased in the reproductive phase. These changes in vitamin E testis and plasma could be explained with steroidogenesis facilitation tissue remodeling and synthesis of collagen that attends the testis-cycle. Furthermore, our results provide an indirect evidence of vitamin E involvement during the gonadal development of this group of vertebrates, as already reported in mammals.

Studies are in progress to define the different antioxidants role during steroidogenesis for the improvement of *Dicentrarchus labrax* reproductive performance.

Respiratory chain-dependent generation and release of superoxide anion and hydrogen peroxide into the mitochondrial intermembrane space

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H_2O_2 diffusing out of mitochondria is believed to originate from the mitochondrial matrix, where it is formed by O_2.- disproportion catalyzed by Mn SOD residing in the matrix. However, the Q cycle also predicts O_2.- release into the intermembrane space, and since a Cu,Zn SOD isoform resides in the intermembrane space, H_2O_2 may also originate from the intermembrane space as well as the matrix. To address this issue, mitoplasts were prepared from isolated mitochondria by digitonin treatment to remove portions of the outer membrane and mitochondrial Cu,Zn SOD. EPR anaylsis of antimycin-supplemented mitoplasts revealed the formation of a DMPO-OH adduct, originating from a spontaneous decay of a DMPO-superoxide adduct. This signal was (a) abrogated by SOD, (b) competitively decreased by exogenous ferricytochrome c, and (c) broadened by the membrane impermeable spin broadening agent chromium trioxalate. Similarly, H_2O_2 production in mitoplasts was enhanced by addition of exogenous SOD, all confirming that O_2.- was being produced towards the cytoplasmic side of the inner membrane. Co-treatment of mitoplasts with myxothiazol and antimycin A- thereby inhibiting the oxidation of ubiquinol to ubisemiquinone- inhibited O_2.- and H_2O_2 production, strengthening the notion that ubisemiquinone autoxidation is a major pathway for O_2.- release into the intermembrane space. The results suggest that intermembrane space along with the matrix is a source of O_2.- and H_2O_2 in mitochondria.

The structure activity relationship the peroxynitrite scavenging by flavonols

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Flavonoids, present in e.g. fruits, tea and wine, can protect against oxidative stress. Indeed, some of these flavonoids have been identified as excellent peroxynitrite scavengers. We have found evidence that there are at least two different pharmacophores in the flavonol structure that can be held responsible for this activity i.e. the catechol moiety of ring B and the hydroxyl group at the 3 position. To assess the influence of the latter group, a flavonol with only one hydroxyl group at the 3 position was synthesized (TUM 9761). Surprisingly, this compound displayed a very poor antioxidant activity 1. We expected an important role for the 3-OH because of the high activity of 3,5,7-trihydroxyflavone compared to 5,7-dihyd roxy-flavone. A possible explanation is that the reactivity of the 3-OH group of the flavonol is probably influenced by other substituents. This was first examined in substituted phenols. A strong correlation was found between the electron donating effect of the substituent and the peroxynitrite scavenging activity. This SAR was extrapolated to the more complex flavonoid structure. Electron donating moieties (e.g. OH-, CH3-, or OCH3-groups) an even number of C-atoms away of the 3-OH group enhance the peroxynitrite scavenging activity. This explains the increased activity of 3,5,7-trihydroxyflavone compared to TUM 9761, with only an OH-group at the 3 position. This SAR is confirmed using newly synthesized compounds. This SAR can be used to identify the reactive center in existing compounds and to design new excellent peroxynitrite scavengers.

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Comparison of mitochondrial oxygen radical production and energetic coupling in polar and temperate bivalves

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Oxygen free radicals (ROS) are common by products of cellular respiration and mitochondria are regarded as the major cellular source of these hazardous active oxygen species. Mitochondrial respiration in a given species increases at higher temperatures, however, adaptation of an animal or a population at permanently or even seasonally low environmental temperatures can lead to a compensational increase of mitochondrial capacities, substrate oxidation and oxygen turnover (review by Guderley 1998, in: Cold Ocean Physiology, Pörtner & Playle (eds.):58). We have studied mitochondrial respiration, energetic coupling to phosphorylation and production of ROS in mitochondrial isolates of the temperate bivalve *Mya arenaria* (Myoidea) and the antarctic clam *Laternula elliptica* (Laternulidae). Both clams are comparable in size and share the filter feeding nutrition mode in the sediment water interface. While the habitat temperature of *Mya* may vary from winterly sub zero temperatures to above 15°C in summer, *Laternula* experiences rather constant temperatures between –2° and +1°C. Experimental warming resulted in an increase of state 3 and state 4+ respiration in mitochondrial isolates from both clams. Highest RC values were found at 15°C in *Mya*, while at higher temperatures mitochondrial coupling decreased significantly and ROS release doubled between 15 and 25°C. ROS production amounted to 2-3% of total oxygen consumption in state 3 (0.3-0.5 nmol ROS /mg protein min) and to 6% in state 4+ (with oligomycin). *Laternula* mitochondria were optimally coupled at 1°C and uncoupled above 7°C. Comparative data on *Laternula* ROS formation will be presented on the poster.

L-arginine rescues decreased erythropoietin gene expression by stimulating GATA-2 with NG-monomethyl L-arginine

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It has been reported that NG-monomethyl L-arginine (L-NMMA) was undetectable in non-uremic subjects, but markedly elevated in uremic subjects. Based on this observation, we hypothesized that L-NMMA is a candidate uremic toxin responsible for the renal anemia. However, the function of L-NMMA in mediating Epo gene expression has not been elucidated. Since L-NMMA functions as an inhibitor of NOS, it was expected that it would suppress the production of NO and cGMP. GATA transcription factors have been demonstrated to bind to the GATA element in the Epo promoter and negatively regulate Epo gene expression. In our previous study, we found that L-NMMA decreased the expression of NO and cGMP and increased the expression of GATA-2 mRNA and levels of GATA-2 binding activity, thereby inhibiting the Epo promoter activity and causing a decrease in the expression level of Epo protein. In the present study, we examined the possibility of the use of L-arginine as a supportive therapy for the renal anemia. Incubation for 24 h with L-NMMA under hypoxic conditions showed an 80 % inhibition of Epo, but this inhibition was recovered by the addition of L-arginine. Hypoxia induced the secretion of NO, but the addition of L-NMMA inhibited this induction, though this inhibition of NO by L-NMMA was recovered by the addition of L-arginine. Hypoxia induced the secretion of cGMP, but the addition of L-NMMA inhibited this induction, though this inhibition of cGMP by L-NMMA was recovered by the addition of L-arginine. L-NMMA induced the binding activity of GATA-2 under hypoxic conditions. This binding activity was inhibited by

the addition of L-arginine. L-NMMA induced the GATA-2 binding activity, but the addition of cGMP inhibited hypoxia-induced or L-NMMA-induced GATA-2 binding activity in a dose-dependent manner. The results of an *in vivo* mouse assay revealed that L-NAME (an analogue of L-NMMA) inhibited the expression of Epo, but this inhibition of Epo expression by L-NAME was rescued by pretreatment with L-arginine. These findings suggest that L-arginine could be used to treat the renal anemia.

Mechanisms of Oxidative Damage in Occular Preservative Action

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Oxidants are commonly used as chemically acting preservatives in solutions for occular application. Ideally, preservatives should prevent microbial growth while being relatively undamaging to mammalian cells. The effect of several preservatives and oxidants on four microorganisms and two mammalian cell lines was studied, with the aim of determining the differences in resistance between the cell types, and the mechanisms responsible for this. Cultured rabbit corneal epithelial cells (RCE) and human conjunctival epithelial cells (WKD) were compared with *Candida albicans, Pseudomonas aeruginosa, Staphylococcus aureus and Alternaria spp*. *Candida* had the highest native intracellular glutathione (GSH) while *Staphylococcus* and *Pseudomonas* had none. The oxidants investigated were *tert*-butylhydroperoxide (t-BHP) and hypochlorous acid. The preservatives tested were benzalkonium chloride (BAK) and PuriteTM. In cell viability experiments BAK was found to be very effective at killing microorganisms, but was damaging to mammalian cells. Purite was also very good at decreasing bacterial and fungal viability, but was much less damaging to epithelial cells, especially over 1-2 hours. The microorganism with overall resistance to the oxidants was *Candida*, and of the mammalian cells RCE was slightly more resistant than WKD. The resistance did not correlate with native antioxidant levels alone. Oxidative treatments lowered the total intracellular GSH in all organisms, with the exception of Puite treatment in *Alternaria* which had no effect on GSH levels. Increased GSH in the media was also observed and may be due to the export

GSSG at cytotoxic levels, or increased cell membrane permeability with cell killing. In conclusion, in eukaryotic organisms resistance to oxidising preservatives was related to the cell's ability to maintain intracellular GSH during treatments, rather than the intracellular concentration per se.

Prevention of multiple organ failure caused by cerebral oxidative damage by traditional Chinese medicine

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It is widely known that reactive oxygen species (ROS) is implicated in complex pathophysiological conditions such as lifestyle- and/or age-related diseases. Brain is essentially weak against oxidative insults because of its low level of physiological defense system including small molecular antioxidants and antioxidant enzymes. In fact, ROS is known to be involved in brain neurodegenerative diseases such as Alzhymer and Parkinson diseases. However, antioxidant protection of brain oxidative damage is not successfully attained by the use of a single antioxidant. Traditional Chinese herbal medicines (TCM) have been successfully used for treating complex disorders. Thus, it is interesting to study the effect of antioxidant TCM on diseases related to oxidative stress. We previously studied the effect of TCM on oxidative damage in rat brain after cerebral ischemia-reperfusion. In the course of the study, it was found that the brain damage accampanied multiple organ failure. Here, we studied further the relationship between the brain and other organs damages after cerebral ischemia/reperfusion, and preventive effect of SMS on these tissues injury. SMS decoction was first granulated with Neusiline as an additive in order to avoid the potential variability of decoctions. The ischemic condition was set by bilateral carotid artery occlusion. After recirculation of cerebral blood flow, both TBARS formation and glutathione peroxidase (GPX) activity were determined as the index of oxidative damage in tissues. We first examined the effect of reperfusion periods after ischemia both on brain and other organs. After ischemia/reperfusion, TBARS formation increased in the brain with reperfusion period. At the

same time, the TBARS formation also increased in other organs, especially in spleen. SMS preadministration inhibited the TBARS formation almost completely in all tissues examined. On the other hand, GPX activity decreased after ischemia/ reperfusion in some tissues but increased in other tissues such as heart and the changes were dependent on reperfusion period. SMS was found to recover the GPX activity to the normal level in all tissues.

Reactivity of anthocyanins toward AAPH radical and peroxides : evaluation by CZE

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Previously, we found from the CZE study (1), that the acid hydrolysis of anthoycanin was governed primarily by the structure of conjugated sugar but not by the aglycon structure (2). In the present study, the reactivity of 15 kinds of anthocyanins in Bilberry was further examind toward AAPH radical, hydroperoxide and t-butylhydroperoxide using the CZE method. From the kinetic decomposition of each anthocyanin, the structure-reactivity relationship was evaluated among the anthocyanins. First, the bleaching of anthocyanin absorbance was measured at 520 nm in the presence of these species. The bleaching rate was found in the following order, H_2O_2 >t-butylhydro-peroxide>AAPH. Then the reactivity of each anthocyanin was more precisely determined by the kinetic decrease of each anthocyanin peak in the CZE electrophoretogram. It was revealed that the reactivity toward AAPH radical was governed primarily by the aglycon structure and not by the type of conjugated sugar. Delphinidins having three hydroxyl group in the B ring of the aglycon was the most reactive to AAPH radical among all anthocyanins, and then cyanidins followed, which has two hydroxyl group in the B ring. When the reactivity was compared among the anthocyanins having the same number of hydroxyl group (free and/or methylated) in the B ring, it was revealed that methylation of the hydroxyl group resulted in the reduction of the reactivity dependent on the extent of methylation. It was thus suggested that the process of anthocyanin decomposition by AAPH radical is

initiated by the proton extraction from the phenolic OH in the B ring. As for the reactivity of anthocyanins toward hydroperoxide, cyanidins was the most reactive and then, followed by delphinidins. However, the reactivity was also determined primarily by the aglycon structure as was seen toward AAPH radical. The reactivity of anthocyanins toward t-butyl-hydroperoxide showed almost the same trend as hydroperoxide. From these studies, it was concluded that the anthocyanins react differently to peroxides and AAPH radical. It was suggested that the peroxides attack the C-2 position of the anthocyanin's A ring rather than the B ring which is the site of AAPH radical attack.

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Increased age-related hepatocellular susceptibility to *tert*-butylhydroperoxide: reversal by (*R*)-α-lipoic acid

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The objective of this study was to determine whether hepatocytes isolated from old rats were more susceptible to *tert*-butylhydroper oxide (tBH) than cells from young animals. Cells were isolated from young (2 mo) and old (24 mo) F344 rats and subjected to oxidative insult using tBH (0, 400, 800 µM). Viability was monitored over a 2 hr time course using LDH leakage. GSH and GSSG levels were measured using HPLC. Results showed that cells from old rats were markedly more susceptible to tBH compared to young. The LC50 for young rats was 786 $\pm$ 14 µM versus 395 $\pm$ 38 µM for cells from old rats, a significant decline ($p < 0.001$). In part this increased susceptibility may be due to a decline in GSH levels and attendant loss of GSH-dependent detoxication mechanisms. Addition of either 400 or 800 µM tBH caused a significant loss of cellular GSH and lower GSH/GSSG ratios 15 min after addition; this loss returned to baseline values 90 min after tBH in cells from young but not old rats. Mitochondrial GSH levels were also more affected by tBH than cytosolic GSH. Feeding old rats with lipoic acid (LA) for 2 weeks reversed the age-related increased susceptibility to tBH. The calculated LC50 of tBH for cells from old rats supplemented with LA was no longer different ($p=0.15$) from that calculated for cells from young animals. Moreover, LA treatment reversed the age-related GSH decline in both the cytosol and mitochondrial fractions. These results indicate that LA may effectively reverse the age-related susceptibility to xenobiotic compounds via maintaining effective GSH concentrations.

Antioxidant effects of a novel isonitrile antioxidant on LDL oxidation and glutamate induced cytotoxicity

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Oxidative stress has been implicated in degenerative processes and many other diseases. Recently, much attention has been paid to natural antioxidants as a defense against oxidative stress.

A novel isonitrile antioxidant named KRIBB695 was purified from a fungal metabolite. The antioxidant effects of KRIBB695 on LDL oxidation and the protective effects on glutamate induced neuronal cell death were investigated. The kinetic study of LDL oxidation resulted in a dose-dependent inhibition by KRIBB695 of the Cu2+- and AAPH-mediated conjugated diene and lipid peroxide formation. The lag times for Cu2+-mediated conjugated diene and lipid peroxide formation were doubled by the treatments of 0.3 μM and 1.0 μM KRIBB695. Treatment with 2.0 μM KRIBB695 resulted in a 50% decrease in glutamate-induced cytotoxicity in N18-RE-105 neuronal cells. KRIBB695 inhibited rat liver micrsomal lipid peroxidation with an IC50 of 1.3 μM. The results suggest that the inhibition of LDL oxidation and glutamate-induced cytotoxicity by KRIBB695 is mediated through its free radical scavenging properties.

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Dietary antioxidants and the risk of non-insulin dependent diabetes mellitus

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The preventive effect of dietary antioxidants against non-insulin dependent diabetes mellitus (NIDDM) has been scarcely studied. Dietary intake of carotenoids and vitamins C and E was studied for its prediction of NIDDM in a Finnish cohort based on 4344 men and women, aged 40-69 years and free of diabetes at baseline. Food consumption during the previous year was determined by a dietary history interview. The intake of vitamin C, 4 tocopherols, 4 tocotrienols and 6 carotenoids was determined. An index of total antioxidant intake was calculated as a combination of all 15 compounds considered. During a 23-year follow-up, a total of 227 male and 165 female NIDDM cases occurred. Total carotenoid intake was significantly inversely associated with subsequent incidence of NIDDM in women but not in men. The relative risks (RR) between highest and lowest quartiles of carotenoid intake, adjusted for potential confounding factors, were 0.58 (95% confidence interval (CI)=0.34-0.98) and 0.89 (CI=0.52-1.50), respectively. The association in women was mainly due to alpha- and beta-carotene and lutein. A significant inverse gradient was observed between total vitamin E intake and NIDDM risk in women (RR=0.68, CI=0.42-1.10, P for trend=0.01) but not in men (RR=0.82, C I=0.45-1.49, P=0.59). The association was mainly due to alpha-, gamma-, and delta-tocopherol and gamma-tocotrienol. Intake of vitamin C was not associated with incidence of NIDDM. Both men and women with a higher antioxidant index level had a reduced risk of NIDDM. The relative risk were 0.41 (CI=0.24-0.72) and 0.52 (CI=0.31-0.87), respectively. These findings of a reduced risk of NIDDM at higher antioxidant levels have to be confirmed in other populations.

Flavonoids including anthocyanidins in foods and their contribution to the average dietary intake in Finland

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Contents of 24 most commonly consumed flavonoids(FLA) were determined in representative food samples consumed in Finland. Altogether 377 samples of most commonly consumed fruits, beverages, vegetables and wild and cultivated berries in Finland were collected in a representative way. Subsamples collected were pooled and homogenized in a blender to make 92 final samples which were analyzed for 24 most commonly existing FLA employing various hydrolyses for the breakdown of glycosides. Following HPLC separation the FLA were identified and quantified by diode array detector (DAD) and an eight channel electrochemical coulometric array detector (CAD); Esa, Inc. USA.) (1). An in-house flavonoid reference matrial was used for the analytical quality control (1).

The total average intake of flavonoids in Finland based on 1997 average food consumption figures was 94.4 mg/d. Berries contributed the most, (38 mg/d .= 40.2%) fruits 37 mg/d (39 %) followed by tea, wine and other beverages (altogether 15.6 mg/d = 17 %), vegetables (2.9 mg/d. = 3.1%) to the total intake. Among the most important flavonoids hesperetin contributed most, 28.3 mg/d. (30 %) to the total average intake. Other major flavonoids were cyanidin, 16 mg/d.(17 %), delphinidin 9.8 mg/d (10.4%), naringenin 8.3 mg (8,8 %), then quercetin 7.0 mg (7.4 %) and epigallocatechingallate 3.6 mg (3.8%). It is concluded that the average intake of flavonoids in Finland may be high enough to have a significant contribution to the antioxidative status of humans. Furthermore, high flavonoid contents are concentrated on fairly few foods resulting in great variation in individual intakes.

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Picomole quantification of deuterium-labeled & unlabeled α– and γ-tocopherols by liquid chromatography-mass spectrometry

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A liquid chromatography-mass spectrometry (LC-MS) atmospheric pressure chemical ionization (APCI) method for quantification of deuterium labeled (d3 *RRR*-α-, d6 *all rac* α- and d2 *RRR*-γ-tocopherols) and unlabeled (d0-) α- and γ-tocopherols following routine vitamin E extraction from biological samples has been developed. Alpha- and γ-tocopherols were separated by LC on a 75 mm C18 reverse phase column using 100% methanol at 1 mL/min. The total run time was 4 minutes. Following negative ionization, each of the tocopherols was detected at its *m/z* of [M-1]. using single ion recording. Fragments produced by increases in cone voltage were similar to those we previously observed using LC-tandem-MS, thereby verifying compound identification. The α-tocopherol detection limit was 10 pg (23 fmol); for routine analysis responses were linear from 0.5 to 25 ng (1.25 to 62 pmol). Detection of γ–tocopherol was twice as sensitive apparently due to greater γ–tocopherol ionization efficiency. The precision of 20 repeated injections was 6-8% for each of the forms injected. To assess accuracy, plasma samples (n = 18) were collected from a subject during and after consuming daily supplements (75 mg each of d3 *RRR*- and d6 *all rac*-α-tocopherol acetates) for one week. The sum of labeled and unlabeled α-tocopherols estimated by LC-MS in these samples was comparable to the α-tocopherol concentrations measured using electrochemical detection (R2=0.9715). In summary, the described method is 10–fold more sensitive than our previous method using LC-tandem-MS; 100-fold more sensitive than using LC with electrochemical detection; and simpler than previous gas chromatography-MS methods in that derivatization of the tocopherols is not required.

Induction of cell death by lysosomotropic detergents

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Controlled lysosomal rupture was initiated in lysosome-rich, macrophage-like cells by the synthetic lysosomotropic detergent, O-methyl-serine dodecylamide hydrochloride (MSDH). When MSDH was applied at low concentrations, resulting in partial lysosomal rupture, activation of pro-caspase-3-like proteases and apoptosis followed after some hours. Early during apoptosis, but clearly secondary to lysosomal destabilization, the mitochondrial transmembrane potential declined. At high concentrations, MSDH caused extensive lysosomal rupture and necrosis. It is suggested that lysosomal proteases, if released to the cytosol, may cause apoptosis directly by pro-caspase activation and/or indirectly by mitochondrial attack with ensuing discharge of pro-apoptotic factors.

Age-related decline of Vitamin C uptake in isolated rat hepatocytes

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The objective of this study was to determine whether characteristics of Na+-dependent Vitamin C (Vit. C) uptake into cells was altered with age and thus contributes to the apparent decline in mammalian tissues from old animals. Hepatocytes were isolated from young (3-4 mo.) and old (24-26 mo.) F344 rats. Cells (106/mL) were incubated with Vit. C (100µM) and uptake was monitored over time using HPLC / ECD. Results showed a significant ($p < 0.01$) age-related decline in Na+-dependent Vit. C uptake. Cells from old rats exhibited an initial rate of 0.082 nmol/min/mg protein versus 0.108 nmol/min/mg protein in cells from young rats. Intracellular Vit. C levels reached a steady-state in cells from both young and old rats at approximately 20 min following addition of exogenous Vit. C; however, the overall accumulation was significantly less in cells from old animals (3.40 ± 0.19 nmol/mg protein) when compared to cells from young animals (6.00 ± 0.70 nmol/mg protein). Moreover the alterations in Vit. C transport characteristics in old cells could be overcome by increasing concentrations of Vit. C added to the extracellular medium. These results suggest that both the rate and KM of Na+-dependent Vit. C transport becomes significantly altered with age, but increasing exogenous levels of Vit. C can overcome these age-related changes.

Genes Involved in Redox Regulation and in Xenobiotic Metabolism as Assessed by Microarrays : E.G. of Mesothelioma and Mesothelial Cells

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Asbestos is responsible for mesothelioma which is a malignant tumor of pleura. In order to better understand the trans-formation process of pleural cells, we compared the gene expres-sion of mesothelial and mesothelioma cells. Using cDNA microarray we assessed expression levels of more than 6,500 genes. Data analysis allowed us hierarchical classification of genes of known function by enzyme, function and pathway clusters and led to characterize both malignant and normal phenotypes. Interestingly, of the fewer than 300 genes that differed between cell lines, most functioned in *i*) macromolecule stability, *ii*) cell adhesion and recognition, *iii*) cell migration, *iv*) extended cell division, *v*) antioxidant defences, and *vi*) xenobiotic resistance. Interestingly, some genes involved in redox regulation like proliferation associated gene A, Cu/Zn superoxide dismutase, and the annexin/thioredoxin couple showed an increased expression level in mesothelioma cells. About 30 genes were screened in the xenobiotic resistance cluster: 8 belonged to the cytochrome P450 family and 4 were glutathione S-transferase encoding genes. All of those genes displayed a moderate or high expression level in both cell lines indicating the intrinsic ability of those cell lines to detoxify xenobiotics. Two genes were involved in the inactivation of chemotherapeutic drugs and were overexpressed in mesothelioma cells: bleomycine hydrolase and dihydropyrimidine dehydrogenase. Genes involved in DNA integrity and repair like GADD45A, nibrin, cyclin H and cdk-7, all induced by DNA lesions, were also upregulated in mesothelioma cells. This peculiar gene profile may explain the unusual resistance of mesothelioma cells to chemo- and radiotherapy. Thus microarray is a powerful tool in understanding human tumour biology and in proposing *à la carte* treatments to patients.

α-Lipoic acid modulates intracellular thiol redox status in 3T3-L1 adipocytes: effects on insulin receptor activation and glucose uptake

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Although the insulin signaling pathway appears to mediate _-lipoic acid (LA) induced glucose uptake in 3T3-L1 adipocytes and L6 myotubes, the molecular target of R-LA action and the nature of the interaction between R-LA and its putative target is unknown. We investigated the mechanism of R-LA action on glucose uptake in 3T3-L1 adipocytes. Pretreatment of 3T3-L1 cells with R-LA for 30 min dose dependently increased glucose uptake in a wortmanin and cytochalasin B sensitive manner. Preincubation of cells with R-LA for various times revealed a biphasic effect of R-LA on glucose uptake; at early time points (30 min-6 h) R-LA stimulated glucose uptake whereas 12-48 h pretreatment inhibited glucose uptake. Analysis of the oxidized and reduced content of LA in the cells and medium showed that >90% of lipoic acid present was in its oxidized form. All oxidized forms of LA (S-, R-, and racemic LA) stimulated glucose uptake whereas the reduced form, dihydrolipoic acid, was ineffective. Measurement of intracellular GSH levels in adipocytes showed that at early time points (before 12 h) there was no change in the level of GSH while longer pre-incubation (24-48 h) of cells with R-LA significantly increased intracellular GSH. Pretreatment of adipocytes with R-LA also increased intracellular peroxide levels at early time points (30 min - 6 h); this was time dependently diminished to baseline levels at the later time points (12-48 h). Moreover, R-LA stimulated autophosphorylation of immunoprecipited insulin receptors from 3T3-L1 adipocytes.

These results demonstrate that i) adipocytes have a low capacity to reduce R-LA and that the oxidized form of lipoic acid is responsible for elevation of glucose uptake, ii) R-LA modulates glucose uptake by changing the intracellular oxidant and thiol redox status, and iii) that the insulin receptor is a potential target molecule for the redox modulatory action of R-LA. Hence, it is suggested that the mechanism by which R-LA stimulates glucose uptake is to oxidize critical thiols of the insulin receptor, resulting in receptor autophosphorylation, thereby increasing glucose uptake.

Antioxidant activity of α-n–acetyl-γ-hyroxy-l-arginine, an unique component in the human placenta

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α-N-Acetyl-γ-hydroxy-L-arginine (Ac-OH-Arg) is a unique component existing only in the placenta, which was first isolated and identified in the human placenta by Mori et al. in 1969. However, no physiological function of Ac-OH-Arg in the placenta has been elucidated until now. In this study, free radical scavenging activities were investigated using an ESR (JES-FR30) with spin trap DMPO for .OH and O_2.-, and [(MGD)2Fe2+] for NO.. D, L-Ac-OH-Arg was synthesized by Mori et al. (BBA 192: 555, 1969). EPC-K1 and SOD standard kit were used as a standard for .OH and O_2.- respectively. Ac-OH-Arg showed potent .OH and O_2.- scavenging activities: 0.39 +/- 0. 05 EPC-K1 μmol-equivalent/mg N=4 and 3.2 +/- 0.7 SOD units-equivalent/mg (N=6) respectively. γ-Hydroxyarginine and N-acetyl arginine also showed almost same scavenging activities against .OH and O_2.-. Ac-OH-Arg effectively scavenged NO. generated from NOC-7. Meanwhile Ac-OH-Arg slightly scavenged DPPH radicals in a lipophilic phase. Ac-OH-Arg was detectable in human placenta from 3 months gestation. Ac-OH-Arg may play a role as an antioxidant for protecting a fetus from free radical injury during gestation.

Acrolein-induced apoptosis in cultured human bronchial epithelial cells. Modulation by vitamin E and ascorbic acid.

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Acrolein is a highly reactive unsaturated aldehyde, identified as the non-cancer hazardous air pollutant of greatest health concern, to which humans are exposed, particularly as a component of cigarette smoke. It has been reported to be a highly selective toxin of the respiratory tract for human and experimental animals. However, little is known about the molecular basis of acrolein-induced cytotoxicity and the ability of antioxidants to counteract the adverse effects of this compound. We investigated the response of human bronchial epithelial cells (HBE1) to acrolein treatment and the modulating effect of vitamin E and ascorbic acid. Our results indicate that acrolein induces a dose-dependent apoptotic response in HBE1 cells, as revealed by annexin V binding and DNA fragmentation, in spite of a strong inhibition of caspases activity. Both antioxidants at micromolar concentrations strongly inhibit acrolein-induced apoptosis. Acrolein induce glutathione depletion and intracellular peroxides generation. Both antioxidants counteract the raise in intracellular peroxides and prevent the oxidative damage to proteins, while glutathione depletion is unaffected. Preliminary data showed that acrolein-induced tyrosine phosphorylation is also inhibited by these antioxidants. Our results showed that both vitamin E and ascorbic acid can modulate the signal transduction pathway triggered by acrolein.

Genistein causes cell cycle arrest via inhibition of p53-independent mechanism in T47D breast cancer cells

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Epidemiological studies suggest that phytoestrogens, such as genistein, reduce the risk of breast cancer. Studies in cancer models support a role for genistein in the modulation of cell cycle checkpoints leading to the inhibition of cell proliferation and apoptosis. Most of these previous studies used tumors containing the tumor suppressor p53. However, 50% of cancers lack p53, thus emphasizing a need for therapeutic agents to target this subset of cancers. We investigated cell proliferation, changes in gene expression and apoptosis using T47D (p53-/-) breast cancer cells. Flow cytometric analysis showed that genistein arrested cell cycle progression in the G2 phase. This was further supported by a time-dependent inhibition of cell proliferation. In addition, we observed a dose-dependent p21 induction, a decrease in Cdc2 activity, and downregulation of the antiapoptotic protein, Bcl-2. Annexin V/ propidium iodide staining revealed a 16% increase in apoptosis after treatment with genistein for 48 hours. These results suggest that genistein causes p21 induction leading to G2 cell cycle arrest with subsequent inhibition of cell proliferation. Also, the suppression of Bcl-2 expression coupled with a negligible effect on apoptosis may imply that alternative mechanisms exist to regulate genistein-induced apoptosis. Collectively, these data show that genistein may elicit an antiproliferative effect via a p53-independent mechanism.

Modulation of antioxidant potential of myoblasts by traditional Chinese medicine, Shengmai-San *in vitro*

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Reactive oxygen species (ROS) has been implicated in cellular injury of myoblasts. Although several studies have been done for the effects of H_2O_2 on skeletal muscles, there are no detailed information about the metabolic changes during muscle cell damage induced by oxidative stress. On the other hand, traditional Chinese herbal medicines (TCM) have been successfully used for treating wide variety of diseases. We have shown that oxidative injury of brain and other organs were prevented by antioxidant TCM, Shengmai-san (SMS) in vivo. It is thus expected that SMS also plays a role in protection of oxidative cell injury of myoblasts.

In the present study, we examined that the effect of SMS on cell viability and glutathione peroxidase (GPx) expression in C2C12 myoblasts, with or without H_2O_2. C2C12 myoblasts were incubated for 1 day with or without SMS in DMEM including 10% FBS. After the pre-incubation, the cultures were switched to serum-free DMEM containing H_2O_2 (0, 0.1, 1, 10 and 100 mM) for 1 hour. Cell viabilities of C2C12 myoblasts were determined by the method of Trypan blue staining. Cell viabilities were seen to decrease with an increase of H_2O_2 concentration. However, SMS (0.33%: v/v) significantly prevented the decline of cell viability. The protective effect of SMS was most prominent in the cultures treated with 1 mM H_2O_2. Thus, immunocytochemical staining was carried out at 0 and 1 mM H_2O_2 with or without SMS by using antibody for GPx. We could observe strong expression of GPx in the cultures treated with SMS compared to either in control or H_2O_2 treated cultures. These results suggest that enhanced resistance of myoblasts against oxidative stress is considered to be due to the augmented expression of intracellular GPx by SMS.

Free radical scavenging activities of new synthetic derivatives containing gluthathione, α-tocopherol, and tryptophan or tryptophan metabolites

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Four newly synthesized compounds containing gluthathione, α-tocopherol, and tryptophan or tryptophan metabolites on succinic acid as the core, i.e., γ-L-glutamyl-S-[2-[[[3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-2H-1-benzo-pyran-6-yl] oxy] cabonyl]-3-N-(A, B, C or D)-3-oxopropyl]-L-cysteinylglycin e sodium salts were investigated for their radical scavenging activities using an ESR (JES-FR30) with a spin trap DMPO for .OH and O_2.- , or [(MGD)2Fe2+] for NO.. A: [2-(5-methoxy-1H-indol-3-yl) ethyl] amino (EMeseroS-GS), B: [2-(1H-indol-3-yl) ethyl] amino (ETptaS-GS), C: 5-hydroxy-L-tryptophan (E5-OHTrpS-GS) or D: L-tryptophan (ETrpS-GS). Hydroxyl (.OH), superoxide (O_2.-) and nitric oxide (NO.) radicals were generated by the Fenton reaction (.OH), hypoxanthine-xanthine oxidase system (O_2.-), or 1-hydroxy-2-oxo-3-(N-3-methyl-aimo-propyl)-3-methyl-1-triazene (NO.). EPC-K1 and SOD standard kit were used as a standard for .OH and O_2.- respectively. All compounds showed potent .OH and O_2.- scavenging activites: 0.43+/-0.07, 0.46+/-0.04, 0.47+/-0.06 and 0.41+/-0.09 EPC-K1 μmol-equivalent/mg, and 2.03+/-0.81, 2.29+/-0.65, 1.91+/-0.56 and 1.13+/-0.37 SOD-equivalent units/mg for EMeseroS-GS, ETptaS-GS, E5-OHTrpS-GS and ETrpS-GS respectively. Using an ESR technique by changing DMPO concentration for the dose-curve, it was shown that all these activities reflect direct radical scavenging. All derivatives showed potent NO. scavenging in the order of:
ETrpS-GS>ETptaS-GS>E5-OH-TrpS-GS >EMeseroS-GS.

Effects of acetyl LDL and probucol on gene expression of human macrophage

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The effects of probucol, which is known as an anti-atherogenic drug, on the expression of 6500 genes in lipid loaded human macrophages were examined using gene chip. The acetyl LDL (AcLDL) with and without probucol was added to macrophages and co-incubated for 72 hr. The macrophages without addition of AcLDL were prepared as a negative control. The total RNA was isolated from cells and analyzed with gene chip. Free cholesterol and cholesteryl esters (CE) which were accumulated in AcLDL-treated cells were analyzed by HPLC. Probucol did not reduce accumulation of CE in AcLDL-treated cells. The treatment of macrophages with AcLDL suppressed the expression of genes encoding sterol regulatory element binding protein (SREBP), methyl sterol oxidase, LDL receptor and fatty acid synthase which are supposed to regulate lipid recruitment or cholesterol synthesis. The genes related with cell proliferation, adhesion, and differentiation were highly expressed in AcLDL-treated cells. Probucol did not affect the expression of these genes. It was found that probucol suppressed the expression of genes regulating immune response in lipid laden macrophage. These results suggest a novel function of probucol by which it may contribute to suppression of development of atherosclerotic lesion.

S-Nitroglutathione produced from peroxynitrite and glutathione activates matrix metalloproteinase (MMP) through unique sulfhydryl modification of MMP

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Oxidative stress causes activation of the precursor of matrix metalloproteinase (proMMP), which is critically involved in tissue injuries. Here we report a novel and unique function of *S*-nitroglut athione ($GSNO_2$) for the regulation of proMMP activation. Purified human proMMP-1, -8, and -9 were incubated with various concentrations of peroxynitrite and GSH, and their activities generated were measured. Intriguingly, GSH strongly potentiated the peroxynitrite-induced activation of all three proMMPs. It is of considerable importance that a transglutathionation to the autoinhibitory domain of the proMMP was found to occur extensively by peroxynitrite as evidenced by fluorographical analysis of the protein labeled with [35S]GSH, indicating that proMMP activation was caused by dissociation of active site Zn-Cys ligand binding in the proenzyme. However, the binding between GSH and the autoinhibitory domain was only partially dissociated by dithiothreitol treatment, suggesting the adduct formation is irreversible and appears not to be through a simple mixed-disulfide formation. MALDI TOF-MS analysis showed that the major product of the reaction of GSH with peroxynitrite was $GSNO_2$ (M/Z of 353). Moreover, synthetic $GSNO_2$ strongly activated proMMPs in a dose dependent manner. It is speculated that a unique sulfhydryl modification of the proMMP such as sulfinyl disulfide (GS(O)SR) may be formed from glutathione sulfinyl nitrite (GS(O)NO) which is generated after intramolecular rearrangement of $GSNO_2$, and thus leading to the proMMP activation.

Mechanisms of stress response signal transduction pathways

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Our working hypothesis is that oxidative stress is a major consequence of the impairment associated with neuronal impairment due to aging or acute trauma. Altered glutathione levels, perturbed neurotrophin signaling and damage to DNA in mitochondria and nuclei are components of responses to both chronic and acute insults to CNS. A corollary of this hypothesis is that oxidative stress has genotoxic and energetic consequences that activate stress response genes via transcription factors such as the NF-κB. The activation of transcription factors, their translocation to the nucleus and their specific binding to cognate DNA sequences regulate expression of gene products essential to neuronal survival and function (stress response genes) such as the superoxide dismutase enzyme, the base excision DNA repair enzyme APE/Ref-1, the Bcl-xL family of proteins, the presenilins and amyloid precursor protein (APP) proteins, and the Choline Acetyltransferase (ChAT) enzyme. We propose that transcription factor binding to cognate DNA sequences is finely tuned by the specificity of the sequence, position within a promoter, and protein-protein interactions with other occupied sites on a promoter. We will discuss this hypothesis in the context of promoter activity of the ChAT, APE/Ref-1, and Bcl-xL genes. Specifically, we will focus on neurotrophin-responsive brain tissues in the aged rat and rats experiencing ischemia/reperfusion as well as a contusion model of spinal cord injury. Supported by NINDS and the Sealy Center on Aging.

Copper-based 'glycochelates' catalyze nitrosothiol decomposition: Possible involvement in the etiology of diabetic neuropathy

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Endothelium-dependent vasodilation is impaired in animals and humans with diabetes and the diminished blood flow to nerves may contribute to diabetic peripheral neuropathy. The impaired vascular relaxation probably reflects reduced bioavailability of endothelium-derived relaxing factor (EDRF), either nitric oxide (NO) or thiol adducts thereof. Transition metal chelators will prevent and even reverse abnormal vascular and nerve function in experimental animal models of diabetes. Even short term administration of one chelator - desferrioxamine - partially corrects diminished arterial relaxation in humans with diabetes. Thus, chelatable transition metals may somehow be involved in defective vascular relaxation in diabetes, perhaps through causing accelerated destruction of EDRF. Upon glycation, proteins such as albumin, collagen and elastin gain an ability to bind Fe and Cu and these 'glycochelates' are redox active. The accumulation of such glycochelates within arterial walls might prevent normal EDRF-mediated dilation through metal catalyzed destruction of NO or NO derivatives such as nitrosothiols. We now report that immobilized glycated protein, when implanted *in vivo*, accumulates bound Cu. Furthermore, both free Cu and Cu-based glycochelates catalyze the rapid destruction of one putative form of EDRF, nitrosothiols. Finally, abnormally high levels of chelatable Cu are present in plasma, tendons and vascular tissues of diabetic mice. Overall, the results support the concept that Cu-containing glycochelates do form in the diabetic condition and may well contribute to the genesis of diabetic peripheral vasculopathy and neuropathy.

Nitrosation, nitration and autoxidation of dopamine

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Catecholamine oxidation reactions could be relevant to physiological conditions and also explain neurotoxicity in Parkinson's disease and aging. In this work, we studied the oxidation of dopamine (DA) promoted by the combination of nitric oxide (NO) and molecular oxygen (O_2).

When a 0.6 mM deoxygenated DA solution (in PBS; pH = 7.4), saturated with NO-gas ([NO] = 1.5 mM), is exposed by effusion to atmospheric air (exposition for 7 min), nitrosation of dopamine by N_2O_3 is observed (λmax = 422 nm). Further exposition to air shows a transformation of the nitroso-DA to its respective nitro-derivative (isosbestic point at 380 nm).

On the other hand, by performing an experiment with a 25 fold lower concentration of NO (i.e., [NO] = 0.06 mM), in air equilibrated PBS (pH = 7.4), dopaminochrome (λmax = 480 nm) was the sole oxidation product detected. In this reaction, NO was consumed (measured with an NO-electrode) suggesting that either it reacts with superoxide after the autoxidation of DA, or, it autoxidizes to NO_2 which would further oxidize DA.

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From ancient remedies to modern therapeutics: pine bark uses in skin disorders revisited

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We studied the effect of French maritime pine bark extract (PBE) on the gene expression profile of HaCaT human keratinocytes using high-density filter arrays. Pine barks have been used for centuries as herbal remedies. The French maritime pine (*Pinus maritima*) bark extract (Pycnogenol®) is a mixture particularly rich in oligomeric procyanidins and other bioflavonoids such as taxifolin, catechin and epicatechin. The expression profiles of both control and PBE-treated cells were determined. Interestingly, PBE was shown to specifically downregulate both calgranulin A and B genes in high-density filter array as it was shown an approx. 22 fold decrease of both gene expressions. This decrease was confirmed in quantitative RT-PCR analysis. Indeed 24 hours following the cell exposure, quantitative RT-PCR using a Lightcycler™ device (Roche Diagnostics) showed 3,100/ng total mRNA calgranulin A copies in control cells *versus* 680/ng total mRNA in PBE treated cells (mean of three independent experiments). Interestingly both calgranulin genes are known to be upregulated in psoriasis and various dermatoses. Actually, high levels of both calgranulin proteins can be detected in abnormally differentiated keratinocytes such as in psoriasis and in various epithelial cell lines. In addition, calgranulins are involved in the calcium-dependent reorganization of cytosqueletal filaments observed in various inflammatory dermatoses. Thus, we propose the use of PBE in human trials on dermatoses.

BH Rihn *et al*., From ancient remedies to modern therapeutics: pine bark use in skin disorders revisited. *Phytotherapy Res*, 15:76-78, 2001.

Ferric nitrilotriacetate induced dna and protein damage: inhibitory effect of a fermented papaya preparation

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The carcinogen Fe-NTA catalyzes the hydrogen peroxide-derived production of free radicals and possibly acts through a mechanism involving oxidative stress. Bionormalizer, a fermented papaya preparation (FPP) is a natural product able to prevent lipid peroxidation in vitro and in vivo. However, little is known about the antioxidant properties of FPP particularly regarding iron-mediated oxidative damage to DNA and proteins. In the present study FPP protected supercoiled plasmid DNA against Fe-NTA plus H_2O_2-induced single and double strand breaks. Similar protective effects of FPP were evident when human T lymphocytes were challenged with Fe-NTA/H_2O_2 and DNA damage was determined by Comet assay. Fe-NTA/H_2O_2 also induced fragmentation of bovine serum albumin (BSA) in vitro and depleted celluar GSH levels in lymphocytes. BSA fragmentation and GSH depletion were dose dependently counteracted by FPP. EPR spin trapping studies demonstrated that protective effect of FPP against Fe-NTA/H_2O_2-induced DNA and protein damage is due to its hydroxyl radical scavenging as well as iron chelating properties.

Does the Generation of Superoxide Anions by NO-Synthase cause endothelial dysfunction in diabetes?

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Background and Aims: The generation of reactive oxygen species (ROS) is discussed to play an important role for impairment of vascular function in diabetes. To elucidate the mechanisms underlying the generation of ROS in diabetes, the influence of glucose and AGE on the release of ROS was studied. **Materials and Methods:** The release of ROS was determined in human endothelial cells (EC) and in porcine coronary strips using the lucigenin method. The production of nitric oxide (NO) was specifically measured by the NO-microelectrode. **Results:** In both models, increasing concentrations of glucose (5 to 30 mM) increased the release of ROS dose dependently. The ROS generation was not only prevented by antioxidants (α-tocopherol, ascorbic acid), but also by L-nitroargini ne (200 µM). The generation of ROS was not observed after removal of endothelium. Even a short term incubation with high glucose (HG, 30 min, 30 mM) abolished the detection of NO completely. The release of NO could be restored by incubation of the coronary strips by antioxidants (45 min) even in the presence of high glucose. **Conclusions:** These data suggest that in the presence of HG (a) the endothelium becomes a source of ROS, (b) that the formation of ROS is dependent on the activity of NO-synthase (NOS), and (c) that a partial uncoupling of the electron transport chain in the NOS complex may cause the enhanced formation of superoxide anions in hyperglycaemia. Changes in the formation of ROS and in the availability of NO have to be expected in long term hyperglycemic states, but also at elevated postprandial glucose concentrations.

Short term high glucose stimulates the generation reactive oxygen species, but eliminates free nitric oxide

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Background and Aims: The generation of reactive oxygen species (ROS) is discussed to play an important role for impairment of vascular function in diabetes. To elucidate the mechanisms underlying the generation of ROS in diabetes, the influence of glucose and AGE on the release of ROS was studied. **Materials and Methods:** The release of ROS was determined in human endothelial cells (EC) and in porcine coronary strips using the lucigenin method. The production of nitric oxide (NO) was specifically measured by the NO-microelectrode. **Results:** In both models, increasing concentrations of glucose (5 to 30 mM) increased the release of ROS dose dependently. The ROS generation was not only prevented by antioxidants (α-tocopherol, ascorbic acid), but also by L-nitroargini ne (200 µM). The generation of ROS was not observed after removal of endothelium. Even a short term incubation with high glucose (HG, 30 min, 30 mM) abolished the detection of NO completely. The release of NO could be restored by incubation of the coronary strips by antioxidants (45 min) even in the presence of high glucose. **Conclusions:** These data suggest that in the presence of HG (a) the endothelium becomes a source of ROS, (b) that the formation of ROS is dependent on the activity of NO-synthase (NOS), and (c) that a partial uncoupling of the electron transport chain in the NOS complex may cause the enhanced formation of superoxide anions in hyperglycaemia. Changes in the formation of ROS and in the availability of NO have to be expected in long term hyperglycemic states, but also at elevated postprandial glucose concentrations.

Cocoa polyphenols inhibit mammalian 15-lipoxygenase-1

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Cocoa polyphenols exhibit antioxidative activities by reaction with reactive oxygen species. A number of such biological activities of procyanidins suggest beneficial effects on the cardiovascular system. We studied the interaction of procyanidins and related compounds with mammalian 15-lipoxygenase-1, which has been proposed to be involved in oxidative modification of low-density lipoprotein. This enzyme is capable of dioxygenating not only free arachidonic and linoleic acids but also phospholipids, cholesterol esters, biomembranes and lipoproteins, thus appearing as a general enzymatic prooxidative agent. Fractions of procyanidin oligomers, isolated from the seeds of *Theobroma cacao*, caused dose-dependent inhibition of isolated 15-lipoxygenase-1 from rabbit reticulocytes at pH 7.4. The inhibitory potencies varied, with the larger oligomers being more active. Thus, the decamer fraction revealed an IC50 of 0.8 µM (or 8 µM if related to the concentration of the monomeric units). Among the monomeric flavanols, epigallocatechin gallate and epicatechin gallate turned out to be the most potent 15-lipoxygenase inhibitors (IC_{50} = 4 µM and 5 µM, respectively). The larger procyanidins also inhibited soybean lipoxygenase-1, suggesting a universal lipoxygenase-inhibitory action. These findings may provide a plausible explanation for the observation that intake of high-polyphenol chocolate decreases the leuko-triene/pro-stacyclin ratio in humans and human aortic endothelial cells as recently reported by Schramm et al. (*Am. J. Clin. Nutr*. 73: 36–40, 2001). Interestingly peroxynitrite, a pivotal inflammatory metabolite, caused complete con-version of epicatechin and procyanidins to other products via oxidation and/or nitration, but did not abolish the inhibitory actions of these compounds on 15-lipoxy-genase-1. Taken together, we propose that lipoxygenase-inhibitory activities of procyanidins and flavanols may contribute to the antioxidative actions of these dietary polyphenols.

Interactions between Ca^{2+}, NO and cytochrome C release during hypoxia/reoxygenation in isolated mitochondria

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Here we demonstrate that Ca2+, even at low micromolar concentrations, is able to induce the release of cytochrome c from isolated rat brain mitochondria. This process was cyclosporin A insensitive and is therefore not mediated by opening of the PTP. Rather, mitochondria did not swell and remained morphologically intact. The intactness of the outer membrane could be illustrated by electron microscopy in presence of respiratory chain inhibitors. Furthermore, the Ca2+-induced cytochrome c release from isolated rat brain mitochondria was not mediated by NO since we were not able to show any sensitivity to inhibitors of the nitric oxide synthase. In contrast rat liver mitochondria showed NO sensitivity during hypoxia/reoxygenation. Active respiration as well as NADH-cytochrome c-oxidoreductase activity were diminished and the formation of protein bound carbonyls was increased in presence of L-Arginine in comparison to incubations in presence of L-NAME after hypoxia/reoxygenation. Under hypoxic conditions, the concentration of NO in the incubation medium increased probably due to the low concentration of oxygen which can react with nitric oxide. Taken together, an increase of cytosolic Ca2+ into the lower micromolar range is suggested to be sufficient to induce the release of cytochrome c whereas the PTP is not required for the induction of apoptosis in neurons. In liver the damage of mitochondria during hypoxia/reoxygenation is, at least partially, mediated.

Cigarette smoke exposure depletes lung vitamin E but not in α-tocopherol transfer protein deficient mice (TTP-/-).

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Animal and human epidemiological studies suggest that sub-optimal levels of vitamin E increase the lung's susceptibility to oxidant injury. Exposure to cigarette smoke (CS) is associated with increased oxidative damage to DNA, lipids and proteins. In smokers, the concentration of α–tocopherol (α-T) in bronchoalveolar lavage (BAL) fluid is decreased while vitamin E quinone is increased. α-T supplementation in mice reduces CS-induced oxidative DNA damage. We used α-T transfer protein (TTP) knockout mice to study the role of α-T in CS–induced lung injury. Mice (n=8 per group: TTP+/+, TTP+/- or TTP-/-) were exposed for 3 days to filtered air or CS (6h/d, particulates 60 mg/m3). Animals were scarified, lungs lavaged and lung tissue was frozen for analysis of α- and γ-tocophe rols. Exposure to CS decreased lung α-T and γ-T concentrations in TTP+/+ or TTP+/- mice compared with air-exposed animals, while the low concentrations in TTP-/- mice were not further decreased by CS exposure. Surprisingly, no increases in markers of inflammation in BAL fluid (cell viability, total cell count, LDH release, total protein, differential cell counts or pro-inflammatory cytokines [IL-1α, MIP-2]) were observed in response to CS-exposure. Because our exposure protocol did not induce inflammatory changes in BAL fluid, we conclude that lung α–T concentrations do not appear to modulate indices of CS respiratory tract damage.

	α-T [nmol/g]		γ-T [nmol/g]	
	Air	CS	Air	CS
TPP+/+	28.60 (23.60 - 37.50)	15.95 (15.30 - 16.60)	1.30 (1.24 - 1.51)	0.75 (0.60 - 0.81)
TPP+/-	30.50 (29.01 - 37.69)	22.70 (14.77 - 27.39)	1.10 (0.81 - 1.24)	0.90 (0.85 - 1.24)
TPP-/-	1.00 (2.11 - 0.98)	2.10 (2.07 - 2.47)	0.20 (0.14 - 0.21)	0.50 (0.40 - 0.48)

Results are given as median and range [nmol/g lung tissue].

Flavonoids protect against oxLDL-induced neuronal apoptosis by attenuating the activation of JNK, c-jun and caspase-3

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Oxidative stress has been associated with neuronal loss observed during neurodegenerative diseases such as Parkinson's and Alzheimer's diseases and age-associated cognitive decline. We have recently demonstrated that oxLDL is capable of eliciting neurotoxicity, including DNA-fragmentation, and that pre-treatment with flavonoids (*e.g.*, epicatechin, kaempferol, cyanidin) protects effectively against these neurotoxic effects.

Major targets for oxidative stress in neurons are the MAP kinases ERK1/2, JNK and p38. OxLDL caused a time dependent increase in the levels of active ERK1/2 and JNK in cultured striatal neurons. OxLDL mediated not only the activation of JNK but also the phosphorylation of c-jun and junD, the cleavage of procaspase-3 and the increase of caspase-3-like protease activity. in neurons. These effects were calcium-dependent and were strongly inhibited by pre-exposure to low micromolar concentrations of epicatechin and kaempferol. Interestingly, one of the major *in vivo* metabolites of epicatechin, 3'-O-methyl-epicatechin, showed no significant difference in protection against oxLDL induced neurotoxicity as well as in the ability to attenuate the oxLDL-mediated activation of JNK, c-jun and caspase-3. The protective effects of the flavonoids could not be mimicked by blocking ERK1/2 activation with the MEK-inhibitor U0126, suggesting that ERK1/2 activation is neither involved in the neurotoxic effects mediated by oxLDL nor the neuroprotective action of flavonoids. Thus, the inhibitory actions of flavonoids on JNK activation may be more important in the mechanisms protecting neurons from death following oxidative stress.

Importance of Gallic Acid as a Marker of Black Tea Intake

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Gallic acid (GA) is found in significant amounts in tea and is a strong antioxidant possessing also antimutagenic and anticarcinogenic activities. We prepared some detailed information about its pharmacokinetics and the bioavailability of GA of black tea in human body. Each of the 10 volunteers drank 200 ml tea brew (Assam; 93% of its GA was in free form) containing 0.3 mmol GA. It was approximately three times more concentrated than normal tea brews. GA was rapidly absorbed but the highest GA concentration observed in plasma (Cmax) was only 2.1±0.2 _mol/L after 1.4±0.2 h. The highest concentration of its metabolite, 4-O-methyl gallic acid (4OMGA) in plasma was not higher than 2.6±0.3 _mol/L (tmax = 1.5±0.3 h), either. Whether this low plasma concentration of GA can have pharmacological activity in the body needs to be investigated. It has already been determined that GA induces apoptosis in various cells at concentrations around 30 _mol/L, and that the effect resulting in cell death of cancer cells is more potent than that against normal cells. In our experiments, **2.1 _mol/L** GA promoted cell growth for human umbilical vein endothelial cells. It did not, however, override the growth controls shown by normal cells. Furthermore, concentrations about 0.3 mmol/L of GA could increase production of methylguanidine (a uremic toxin) from creatinine in isolated rat hepatocytes. However, **2.1 _mol/L** of GA could not show any detectable effect on this reaction.

Although with respect to Ae (total amount collected in urine) more than 60% of GA excreted was metabolized to 4OMGA, there is no evidence whether this metabolite can contribute to the pharmacological effects. Therefore, profound understanding of pharmacological activities of low concentrations of both GA and 4OMGA is highly desirable.

Ubiquitin-independent degradation of oxidized proteins by proteasome

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Oxidatively modified forms of proteins accumulate during aging and in many pathological conditions. Mammalian cells exhibit limited direct repair mechanisms and oxidatively damaged proteins appear to undergo selective proteolysis, primarily by the major cytosolic proteinase, the proteasome. The proteasome exists in at least two different forms - 20S and 26S. The 20S proteasome is the catalytic core, whereas the 26S form contains additional subunits for ATP hydrolysis and poly-ubiquitin recognition. Purified 20S proteasome can degrade oxidized proteins in the absence of ATP and ubiquitin *in vitro*, though it is not clear if the proteasome can function independently of ATP and ubiquitin *in vivo*. The primary aim of this study is to determine if the degradation of oxidized proteins by the proteasome *in vivo* is dependent on either ATP or ubiquitin. We examined the turnover of oxidized proteins in a cell line incapable of carrying out ubiquitin-conjugation. These studies employed a cell line with a conditional mutation for the ubiquitin-activating enzyme E1, which controls the first step of the ubiquitin conjugation pathway. Our results indicate that cells incubated at the restrictive temperature with compromised ubiquitin conjugating activity, are still capable of preferentially degrading oxidized proteins. This ubiquitin-independent turnover of oxidized proteins is mediated by the proteasome as it can be inhibited by lactacystin, a selective inhibitor of the proteasome. The ts20 cells are capable of eliminating oxidized proteins as measured by the carbonyl content, which returns to control levels within 24 hours after treatment with H_2O_2. In separate, *in vitro* assays for ubiqutin conjugation of native and oxidized ferritin, we have seen that progressive oxidation does not promote more ubiquitinylation, but causes a slight decrease in the extent of ubiquitinylation.

Carotenoid breakdown products inhibit mitochondrial respiration

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To study the biological effects of carotenoid oxidation products in suspensions of rat liver and brain mitochondria, mixtures of beta-carotene, retinal, and beta-ionone breakdown products (CBP) were produced by hypochlorous acid-induced oxidation. The oxidation products were analyzed by capillary gas-liquid chromatography and HPLC combined with MS. For evaluation of biological effects of CBP "endogenous" respiration, ADP stimulated respiration, and uncoupled respiration of liver and brain mitochondria were measured after exposure to different CBP mixtures (0.5 to 20 µM). Furthermore, the mitochondrial SH content and MDA formation were detected after treatment. The ADP stimulated oxygen consumption was strongly affected by all CBP mixtures used. In liver mitochondria 20 µM CBP led to up to a 50 - 60 % decrease of ADP stimulated respiration in brain mitochondria. The endogenous respiration was hardly affected. Uncoupled respiration was changed only by beta-ionone and beta-ionone breakdown products. The mitochondrial protein SH content decreased after addition of CBP from 95 to 85 or to 70 nmol SH/mg protein, respectively in presence of 20 µM beta-carotene CBP or retinal CBP in liver mitochondria, and from 70 to less than 60 nmol SH/mg protein in presence of all types of CBP at 20 µM in brain mitochondria. In parallel, a decrease of mitochondrial GSH was observed in presence of CBP. MDA formation is an early event after exposure of mitochondria to CBP. Brain mitochondria were more sensitive than liver mitochondria towards CBP effects. The measured inhibitory effects in mitochondrial respiratory parame-

ters argue for the inhibition of the adenine nucleotide translocator and/or inhibition of ATPase by the CBP. The effects measured already at low concentrations of CBP may have pathophysiological significance. They indicate a potential toxicity of oxidative carotenoid metabolites and/or their biological activity as signal modulators.

Plasma cholesterol oxidation products (oxysterols) in chronic lymphedema

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Cholesterol oxidation products (oxysterols) are commonly found in foods of animal origin and are also produced endogenously in the body. They may serve as a marker for in vivo oxidative stress. Some oxysterols were shown to be cytotoxic at μM concentrations and to exert further biological activities including effects on sphingolipid metabolism, cholesterol homeostasis, platelet aggregation, apoptosis of different cell types, and protein prenylation. Oxysterols are present at high concentrations in atherosclerotic lesions.

In former studies an increased formation of aldehydic lipid peroxidation products in chronic lymphedema was demonstrated. In this study plasma oxysterol levels were measured using gas chromatography in patients with chronic lymphedema using 17 patients with lymphedema (state II or III) as compared to 20 healthy controls. 7-Ketocholesterol (7K) ($p<0.01$), β-epoxycholesterol ($p<0.01$), α-epoxycholesterol ($p<0.05$), and cholestanetriol ($p<0.01$) were increased in chronic lymphedema. The highest difference was found for 7K, being 0.039 ± 0.014 μM in lymphedema and 0.022 ± 0.011 μM in controls. There were no significant differences of 7α-hydroxycholesterol, 7β-hydroxycholesterol, and 20α-hydroxy-cholesterol between patients with lymphedema and healthy controls. Increased plasma levels of oxysterols in chronic lymphedema patients indicate in vivo oxidative stress in this disease. It is suggested that increased oxysterol levels – from lymphatic vessels, in lymphedematous tissue, and in the blood circulation – may contribute to the progressive damage of lymphatic vessels, to atherosclerosis, and furthermore to fibrosclerotic degeneration in these patients.

Increase in nitric oxide- induced oxidative stress by glutathione depletion in primary rat hepatocyte cultures: involvement of low molecular weight iron

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The aim of our investigations was to examine, in primary rat hepatocyte cultures, the effects of a glutathione depletion on nitric oxide-induced oxidative stress, when nitric oxide was produced by NO synthase before the glutathione depletion. For this purpose, cultures were preincubated with lipopolysaccharide (LPS) and γ-interferon (IFN) in order to induce nitric oxide (NO) production by NO synthase. Then, glutathione depletion was performed by exposure of these cells to L-buthionine sulfoximine (BSO). In cultures preincubated with LPS and IFN prior to BSO addition, an increase of oxidative stress was observed, compared to cells treated with LPS and IFN only. Moreover, simultaneously, NO production was assessed by measuring nitrites in the culture medium, dinitrosyl iron complex (DNIC) and mononitrosyl iron complex (MNIC) in intact cells. BSO supplementation had no effect on nitrite levels but led to an increase in DNIC levels and a decrease in MNIC levels. As MNIC and DNIC corresponded respectively to free NO and to NO bound to iron containing molecules, the elevation of DNIC levels suggested us a possible increase in low molecular weight (LMW) iron when BSO was added to cultures pretreated with LPS and IFN. Thus, experiments of LMW iron chelation by deferiprone were performed. Addition of deferiprone triggered a decrease in oxidative stress and DNIC levels. Moreover, measurements of LMW iron levels by electron paramagnetic resonance showed an important elevation of LMW iron levels after 2 hours of BSO supplementation. These results suggested that the increase in nitric oxide-induced oxidative stress by glutathione depletion was mediated by an enhancement of LMW iron.

Identification of new short-chain breakdown products after oxidative degradation of beta-carotene by hypochloride.

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Beta-carotene is widely used as a food supplement and as a prophylactic and therapeutic agent. However, there are still controversies about its potential toxicity after a number of clinical efficiency trials failed. There is some evidence that oxidative breakdown of beta-carotene may lead to the formation of toxic products which might be harmful under certain conditions. In previous experiments we were able to show that a mixture of metabolites after oxidative breakdown of beta-carotene led to a rapid loss of Na+-K+-ATPase activity [Siems, Sommerburg, van Kuijk: Free Rad Res 2000]. Compared to other biologically relevant aldehydes (beta-Apo-10'-carotenal, retinal, HNE) the inhibitory effect of the breakdown product mixture to the enzyme was much greater. In the last years a number of apo-carotenals was already identified as oxidative breakdown products of beta-carotene. However, the damage caused by the mixture of beta-carotene breakdown products led to the assumption that molecules shorter than apo-carotenals might be responsible for the irreversible enzyme inhibition. Therefore, we tried to detect and identify such short-chain products appearing after oxidative breakdown of beta-carotene by hypochloric acid. Identification analysis was carried out by gas chromatography - mass spectrometry. For GC-MS analysis aliquotes of the dried reaction mixture were dissolved in hexane yielding final concentrations of 15µmol beta-carotene-aquivalent. After GC separation products were detected in an ion trap mass spectrometer scanning from 50 to 650 amu with a scan rate of 0.5 sec/scan. Trimethylcyclohexenone, β-cyclocitral, trimethyltetrahydro-naph-

thalene, 5,6-epoxi-β-ionone, and dihydroactinidiolide were found as new metabolites of oxidative breakdown of beta carotene. The discovery of those and further metabolites of carotenoid oxidative breakdown is important for the understanding of the molecular actions and biological effects of carotenoids.

Cellular responses to oxidative stress: oxidative modification of phospholipids

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We have used electrospray mass spectrometry (ESMS) to investigate the effect of oxidative stress on membrane phospholipids in the cultured human cell lines U937 and HL60. This allows intact oxidized phospholipids rather than breakdown products to be observed, providing specific information on the initial fate of membrane components. Cells were incubated with hypochlorous acid (HOCl) or *t*-butylhydroperoxide + Fe2+, and subsequently extracted with chloroform-methanol for analysis by reverse phase HPLC coupled to ESMS. Both phosphatidyl-choline (PC) chlorohydrins and hydroperoxides could readily be observed in cell extracts following treatment with the respective oxidants. Monoperoxides of C34:2 PC and C36:2 PC were observed, consistent with the fact that these were the most abundant di-unsaturated lipids present in the cells. The major products with HOCl were found to be monochlorohydrins of C32:1 PC and C34:1 PC, despite the availability of more unsaturated fatty acyl chains for modification. Further studies with U937 cells showed that the appearance of modified phospholipids was dependent on the severity of treatment, increasing approximately linearly with increasing oxidant concentration. Hydroperoxides could already be detected after 15 minutes, and increased in concentration until approximately 3 hours. Despite the appearance of detectable oxidized phospholipids, the cell lipid profile of both cell types appeared to be relatively resistant to oxidative damage. Overall, this approach offers a good model for studying the involvement of membrane phospholipids in cellular responses to oxidative stress in relation to antioxidant status.

Endothelial cell activation by epoxyisoprostane phospholipids: Evidence for their formation under oxidative stress

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Monocyte recruitment to the vessel wall plays an important role in atherogenesis. We have previously shown that minimally modified LDL and oxidized 1-palmitoyl-2-arachidonyl-*sn*-phospha-tidylcho line (Ox-PAPC), activate human aortic endothelial cells (HAEC) to produce monocyte chemotactic protein-1 and interleukin-8 (IL-8). In the present study we have identified the components of Ox-PAPC that are responsible for the majority of the production of IL-8. We have employed normal phase and reverse phase HPLC/ MS to isolate the various components and tested their ability to induce HAEC to produce IL-8. We found five peaks of the molecules with *m/z* 828.5 (PEIPC isomers) and four peaks of the molecules with *m/z* 810.5 (dehydration products of PEIPC isomers) that were active in inducing IL-8 and were also potent in activating the PPRE in transfected HeLa Cells. Chemical modification of PEIPC with sodium borohydride or epoxide hydrolysis resulted in the loss of activity, suggesting the importance of carbonyl and epoxide groups for the bioactivity. The evidence for the isomeric structures of these oxidized phospholipids was obtained by tandem mass spectrometry analysis. The levels of these phospholipids were increased by 3 to 5- fold when HAEC were exposed to IL-1β for 18 h. These studies identify oxidized phospholipids that induce the production of inflammatory chemokines in endothelial cells and provide evidence for their formation under the conditions of oxidative stress.

Lipid hydroperoxide-induced stress enhances leukocyte-endothelial interaction in the retinal microcirculation

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We evaluated leukocyte dynamics *in vivo* in rat retinal microcirculation following exposure to lipid hydroperoxide (LHP). 1 or 10 μg of LHP (18:2) dissolved in 5μl of sodium borate buffer (SBB, 0.02M) was injected into the vitreous of Brown-Norway rats. Vehicle-treated rats were given SBB only. Control received the same amount of phosphate-buffered saline (PBS, 0.1M). At 6, 12, 24 and 48 hours after LHP injections, we evaluated 1) the flux of rolling leukocytes along the major retinal veins and 2) the number of leukocytes that accumulated in the retinal microvasculature with acridine orange digital fluorography. In LHP (10 μg)-treated rats, leukocyte rolling along the major retinal veins was maximal at 6 hours after LHP injection (111±10 cells/min). No rolling leukocytes were observed in control, vehicle-treated and low LHP(1μg)-treated eyes. However, the number of accumulated leukocytes in LHP (1 or 10 μg)-treated eyes started to increase at 12 hours (26±14 and 152±34 cells/mm2, respectively), and peaked at 24 hours (98±31 and 788±372 cells/mm2, respectively) which was significantly higher than in vehicle-treated eyes and control (53±23 and 44±22 cells/mm2, respectively, $p<0.01$). The present study demonstrates that increased LHP levels in the vitreous enhance leukocyte-endothelial interaction in the retinal microcirculation.

Lipofuscin accumulation in neonatal rat cardiac myocytes: Correlation with oxidative stress and mitochondrial damage

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Lipofuscin accumulation and mitochondrial damage are major characteristics of aging postmitotic cells. Normally, different cells of the same type contain variable amounts of lipofuscin and damaged mitochondria, suggesting that they age at different rates. It is not clear, however, whether both these changes occur in parallel in single cells, and whether they reflect the level of oxidative stress, the main factor of age-related damage. For lipofuscin autofluorescence assessment, we obtained images of live neonatal rat cardiac myocytes (aged 1, 2, 4 and 8 weeks) using laser scanning microscopy (green excitation light). The same cultures were then examined for formation of reactive oxygen species (ROS, by oxidation of dihydrorhodamine-123 or dihydro-ethidium) or for mitochondrial membrane potential (Ψ, by uptake of the mitochondrial tracker JC-1). Comparison between values of lipofuscin, ROS formation and amounts of damaged mitochondria (estimated by low Ψ) in single cells showed a strong positive correlation between all these parameters. This correlation was observed even in young cells (aged 1 or 2 weeks, with minor degrees of lipofuscin accumulation and mitochondrial damage), suggesting the primary role of oxidative stress in both lipofuscin formation and mitochondrial changes. Considering that lipofuscin-loaded lysosomes have limited autophagocytotic capacity1,2 (needed for recycling of damaged mitochondria), the accumulation of damaged mitochondria in old cells also may occur secondary to lipofuscin deposition.

1. Terman A, Dalen H, Brunk UT. Exp Gerontol 1999, **34**, 943-57.
2. Terman A. Redox Report, 2001, **6** (1), in press.

Heavy accumulation of damaged mitochondria in neonatal rat cardiac myocytes following prolonged inhibition of autophagy

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Autophagocytosis is an important mechanism involved in continuous recycling of oxidatively damaged cellular organelles. It includes sequestration of cytoplasmic fragments containing damaged organelles, fusion of sequestered vacuoles with lysosomes, and digestion of the contents by lysosomal enzymes. To gain a better understanding of the role of lysosomes in the recycling of cellular components, we exposed cultured neonatal rat cardiac myocytes to 5 mM 3-methyladenine (3MA), a phosphoinositol-3-kinase inhibitor which suppresses the first step of autophagy. Such treatment resulted in reduction of the number of lysosomes (estimated by uptake of the lysosomotropic weak base acridine orange) and progressive accumulation of damaged mitochondria (with low membrane potential estimated by poor uptake of the mitochondrial tracker JC-1) in the periphery of cells. Electron microscopy (twelve days after initiation of 3MA treatment) showed numerous densely packed mitochondria with different degrees of structural alterations ranging from destruction of single cristae to complete homogenization of matrix and inner membranes. Continuation of 3MA administration resulted in the loss of contractility of the myocytes and their eventual premature death. The results suggest that in highly aerobic cells, such as cardiac myocytes, mitochondria are intensely damaged by oxidative stress, and their autophagocytotic recycling is critically important for life maintenance. Thus, decreased autophagy (such as occurs in lipofuscin-loaded old postmitotic cells1,2) may substantially contribute to mitochondrial damage and cell death.

1. Terman A, Dalen H, Brunk UT. Exp Gerontol 1999, **34**, 943-57.
2. Terman A. Redox Report, 2001, **6** (1), in press.

Reduction of diabetic cataract risk using l-nitroarginine, an inhibitor of no synthase.

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Purpose: The oxidative stress of diabetic hyperglycemic episodes elevates osmotic pressure in the eye, inducing cell damage and protein leakage in the lens. There are several model pathways that have been proposed in cataract formation relating to elevated glucose levels. One pathway in the lens which has not been examined previously is the production of nitric oxide (NO) via nitric oxide synthase (NOS). NO can be converted to peroxynitrite (ONOO) by reaction with superoxide anion. Superoxide is produced during nonenzymic glycation of proteins incubated in elevated concentrations of glucose. ONOO is an extremely damaging reactive oxygen species (ROS). Nω-nitro-L-arginine (L-NA) is an inhibitor of NOS, and can potentially decrease NO formation and thus decrease ONOO formation.

Methods: Rat lenses were incubated with and without L-NA in either normal (5.6 mM) or elevated glucose (55.6 mM) media. Protein levels were analyzed every 24 hours for a total of 8 days and lenses were photographed to document opacification.

Results: 1mM L-NA inhibits the damage which results in protein leakage and opacification caused by elevated glucose levels but causes damage to lenses in normal concentrations of glucose. Lower concentrations of L-NA (0.10 mM) are effective in preventing protein leakage and opacifiction caused by elevated glucose. In normal lenses this did not result in elevated protein leakage although slight opacification was seen**.**

Conclusions: NOS seems to be involved in diabetic cataractogenesis, and NOS inhibitors may be useful drugs to reduce the risk of diabetic cataract. NOS appears to play an important role in the normal lens since strong inhibition in normal lenses can cause opacity and protein leakage.

Effect of lipid peroxidation products on IL-8 gene expression in human bronchial epithelial cells

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Exposure of humans to environmental oxidants such as ozone and tobacco smoke can cause peroxidation of lipids in the respiratory tract. Oxidation of the respiratory tract lipids, including cell membrane lipids leads to the formation of aldehydic by-products, including malondialdehyde, straight chain aldehydes (hexanal) and α,β-unsaturated aldehydes (such as acrolein and 4hydroxy-nonenal) that potentially act as mediators of cell injury. Acrolein (CH 2=CHCHO), a component of combustion present in high concentrations in cigarette smoke, is a strong electrophile and shows high reactivity with nucleophiles, such as sulfhydryl groups. The aim of this study was to investigate if acrolein and hexanal are able to modulate respiratory tract cell expression of the proinflammatory chemokine IL-8. Confluent human bronchial epithelial cells (HBE-1) were incubated for 30 min, with different concentrations of acrolein or hexanal. Acrolein at concentrations ranging from 0 to 25 μM for 6 and 12 hours resulted in a dose-dependent decrease of HBE-1 cell IL-8_mRNA steady state levels. This effect was most pronounced after 12h of incubation, with a decrease of IL-8 mRNA levels to 26, 40 and 79% at 5, 10 and 25 μM acrolein concentrations, respectively. Measurements of HBE-1 IL-8 production (by ELISA) were consistent with the expression results. Incubations of HBE-1 cells with 25 μM acrolein for 12 hours resulted in a 60% decrease in IL-8 release. In contrast, hexanal did not modulate the IL-8 mRNA expression or the IL-8 release in HBE-1 measured by RT-PCR and ELISA respectively. NF-kB is a known promotor for IL-6 production. Our data are thus consistant with findings of others that acrolein inhibits NF-kB. Further studies are needed to show that acrolein down regulates other NF-kB-regulated proinflammatory cytokines (e.g. IL-1 and IL-6) and to more specifically characterize definitive mechanisms of acrolein-related respiratory tract toxicities.

Nitric oxide and superoxide radical production by human mononuclear leukocytes

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Both nitric oxide (NO) and superoxide anion (O_2-) are important mediators of cellular immune response and they are formed as products of the activities of the nitric oxide synthases (NOS) and NADPH oxidase, respectively. In the immune response, NO controls the regulation of diverse processes and it is a reactant for the production of the cytotoxic peroxynitrite (ONOO-). The oxidative metabolism of monocytes and lymphocytes has not been extensively investigated. As a consequence, the aim of this work is to determine the production rates of O_2-, H_2O_2, NO and ONOO- by a preparation of human mononuclear cells, where the major proportion corresponds to lymphocytes (90%).

Mononuclear cells were isolated by Ficoll-Hypaque gradient from blood of healthy human donors. Production of NO, O_2- and H_2O_2, oxygen consumption and luminol-amplified chemi-luminescence were determined at 37°C, before and after stimulation with 0.1 μg/ml phorbol 12-myristate 13-acetate (PMA).

Human mononuclear cells produced in resting state 0.11 ± 0.01 nmol NO/min.106cells and 0.25 ± 0.02 nmol O_2-/min.106cells. When these cells were stimulated with PMA, the production rates increased to 0.20 ± 0.02 nmol NO/min.106 cells and 0.76 ± 0.12 nmol O_2-/min.106cells. Oxygen uptake accounted for the sum of the rates of NO and H_2O_2. The addition of PMA produced a 3-fold increase in the H_2O_2 production rate and in the H_2O_2 steady-state concentration. Chemiluminescence was 13-fold increased by PMA supplementation. Pre-incubation of mononuclear cells with L-NMMA (NOS inhibitor) or L-NMMA and SOD produced a decrease of 43% and 83% in the chemiluminescence signal, respectively, indicating that ONOO- is the main oxidizing species involved.

It is apparent that NO and O_2- production by human mononuclear cells may constitute the basis of intercellular signaling and cell toxicity.

α-Tocopherol inhibits human glutathione S-transferase π

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α-Tocopherol (vitamin E) is the most important fat-soluble, chain-breaking antioxidant. It is known that interplay between different protective mechanisms occurs. GSTs can catalyse glutathione conjugation with various electrophiles, many of which are toxic. The Pi class of GST appears to be specifically vulnerable to oxidative effects. We assumed that antioxidants like α-tocopherol might have a protective effect on GSTπ.

We found that the activity of glutathione S-transferase π is inhibited by α-tocopherol in a concentration dependent manner, with an IC50-value of 0.5 μM. To obtain information on the nature of the inhibition, GST activity was measured with variable concentrations of either CDNB or GSH in the presence or absence of a fixed concentration (0.6 μM) of α-tocopherol. GST shows characteristic Michaelis Menten behavior towards both substrates. We found that α-tocopherol lowered the Vmax values, but did not affect the Km for either CDNB or GSH. This indicates that the GSTπ enzyme is non-competitively inhibited by α-tocopherol.

The implications of the inhibition of GSTπ by α-tocopherol could in principle be far-reaching. It is known that GSTπ is present in the skin. Henderson et al. showed that mice lacking the GSTπ have an increased risk for skin tumorigenesis 1. Interestingly, Mitchel and McCann found that vitamin E is a complete tumor promoter in mouse skin 2. The potent GSTπ inhibition by α-tocopherol found in our study suggests that this promoter effect might be caused by GSTπ inhibition.

1. Henderson et al. *PNAS,* 95(5);5275-5280, 1998.
2. Mitchel, McCann. *Carcinogenesis,* 14(4);659-662, 1993.

Response of mitochondria to oxidant stress: monitoring mitochondrial parameters in permeability transition

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The mitochondial (mt) permeability transition pore (PTP) is reported to play a role in apoptosis. We investigated some well-studied parameters in mt permeability transition (MPT), but included 2 novel parameters in MPT: we determined the conformational changes and damage to mtDNA and mt protein release. **DESIGN**: We exposed isolated rat liver mt to either of, *tert*-butylhydr operoxide, iron or phosphate, to induce MPT. Concurrent with monitoring mt functional analysis (such as mt swelling, Ca2+ release, mtDNA damage and lipid peroxidation), the extramitochondrial buffer was sampled for subsequent protein analysis by mass spectrometry (using tryptic peptide mass fingerprinting of the 1D SDS-PAGE separated species utilizing MALDI-TOF MS). Conformational changes of mtDNA were assessed using purified mtDNA subjected to agarose gel electrophoresis followed by densitometry. Density of the mtDNA bands permitted comparison of the different forms of mtDNA and fragments from experimental mt versus controls. Mt functional parameters were investigated by measuring mt respiratory control ratios and Ca2+ release. The extent of lipid peroxidation was assessed by MDA formation, which was determined by GC-MS. **RESULTS**: Iron exposure resulted in decreased RCR, mtDNA fragmentation, Ca2+ release and large amplitude swelling. Preliminary results of the proteins released by mitochondria indicate that there is a temporal specificity to the appearance of some proteins outside of this organelle. **CONCLUSIONS**: Oxidant stress induces mt respiratory dysfunction and changes in mtDNA conformation. MtDNA conformational changes may play a role in mt permeability transition. Furthermore, the apparent release of mt proteins may be significant to the mechanism of apoptosis.

Involvement of oxidative stress in pentylenetetrazol-induced seizure and kindling of rats

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Various animal models have been used to study pathophysiological and pharmacological aspects of epilepsy. Apart from an acute convulsion model studying clonic-tonic seizures induced by a single dose of chemical stimulant pentylenetetrazol (PTZ) the repeated application of initially subconvulsant doses of PTZ results in a progressive intensification of seizure activity culminating in generalized clonic-tonic seizures, too (so-called kindling phenomenon). Following acute PTZ-induced seizures and the development of PTZ-kindling an enhanced formation of free hydroxyl radicals in rat brain could be demonstrated earlier (Rauca et al., 1999). A causal connection between oxidative stress induced by convulsants and neuronal loss was supposed. It was the aim of the present study to find out, if the generation of hydroxyl radicals is reflected by an increased lipid and/or protein oxidation and changes in the levels of antioxidants in rat brain homogenates and isolated mitochondria following PTZ-induced seizures and kindling. We investigated TBARS and isoprostanes (derived from arachidonic and docosahexaenoic acid) and protein-derived carbonyls as markers of lipid and protein oxidation as well as α-tocopherol and glutathione (GSH, GSSG) in the brains of rats treated with an acute seizure-inducing dose of PTZ and in PTZ kindled animals which were injected PTZ every 48 h, over a period of four weeks. Control animals were treated with NaCl instead of PTZ.

The data confirm the involvement of free radicals in acute PTZ-induced convulsions and PTZ-kindling. Changes in oxidants (protein-bound carbonyls and isoprostanes) and antioxidants (α-tocopherol, GSSG/GSH) are more pronounced in mitochondria than in homogenates. Differences between kindled and acutely convulsing rats are generally not very large. Investigations in specific regions of the brain may be important for future work.

Rauca, C., Zerbe, R. and Jantze, H. Brain Research 847 (1999) 347-351

The optimal time and dosage of nicotinamide therapy following transient focal cerebral ischemia in Wistar rats

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Our laboratory has previously confirmed that Nicotinamide has powerful neuroprotection on oxidative stress in brain induced by 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydro-pyridine (MPTP) or t-butylhydroperoxide (tBuOOH). Nicotinamide (NM) also protects brain damage in both transient focal ischemia (IS) and reperfusion (RP) in rats and global model in mice. However, suitable considerations for dosage and optimal time after IS/RP are lacking where NM can be used efficaciously to treat stroke in clinical practice. Transient focal cerebral IS was induced by MCAO for 90 minutes, followed by RP for either 24 hours or 48 hours in Wistar rats. Different doses of NM were injected IP when RP occurred and after 24 hours the brain samples were taken. In another 48 hours design, 500 mg/kg NM was administered at the different time after onset of RP. Neurological finding scores were recorded, and infarct volumes of brain were measured. In contrast to the cases treated with the vehicle, the neurological deficit scores and infarct volume were greatly reduced at 24 hours of RP by treatment with NM. ED50 was 277.6±69.05 mg/kg (P=0.95). The administration of NM during different stages after 90 min of IS of MCAO and at 48 hours of RP, it was found that 500mg/kg NM injected after the first 6 hours of RP could effectively inhibit the development of brain damage.

Conclusions- The nuclear enzyme poly(ADP-ribose) polymerase (PARP) plays a key role in DNA repair in stroke. Excessive PARP activity consumes NAD leading to energy depletion and exacerbation of neuronal damage. As an inhibitor of PARP, NM promotes energy supply. The present results suggest that early application of NM with a suitable dosage significantly ameliorates brain injury after transient focal brain IS/RP.

Iron metabolism and erythrophagocytosis by macrophages in atherogenesis *in vitro* and *in vivo*

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Iron has been implicated in atherogenesis through its capacity to initiate oxidative stress, which is supported by some epidemiological and many experimental studies. We have proposed previously that lysosomal iron, partly released from the iron-laden macrophages, may contribute to lipoprotein oxidation, ceroid formation, and lipid-induced cellular injury. Here we further studied perturbed iron metabolism in macrophages, their interaction with lipoproteins, and the origin of iron accumulation in human atherosclerotic lesions. We found that iron uptake into macrophages, via transferrin receptors or scavenger receptor-mediated erythrophagocytosis, leads to accelerated synthesis of ferritin at both mRNA and protein levels. The binding activity of iron regulatory proteins was increased by desferrioxamine and decreased by hemin and iron salts. Both direct uptake of iron and erythrophagocytosis also resulted in increased cellular iron. Iron-laden macrophages secreted both iron and ferritin into the culture medium. The secretion of iron was enhanced by lipoproteins, whereas the secretion of ferritin was stimulated by oxidized low-density lipoprotein (oxLDL) and inhibited by high-density lipoprotein (HDL). In human atherosclerotic lesions, hemoglobin and ferritin were co-localized in macrophage-rich areas, thereby further linking erythrophagocytosis to iron-accumulation in human atheroma. We conclude: (*i*) iron-uptake and erythrophagocytosis lead to increased ferritin expression and exocytosis; (*ii*) oxidised LDL and HDL have different effects on such exocytosis; and (*iii*) erythrophagocytosis is a major source of iron deposition in human atheroma. These events may provide an underlying explanation to processes that lead to oxidative cellular damage and lipid peroxidation during atherogenesis.

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Recent advances in nitric oxide sensor design

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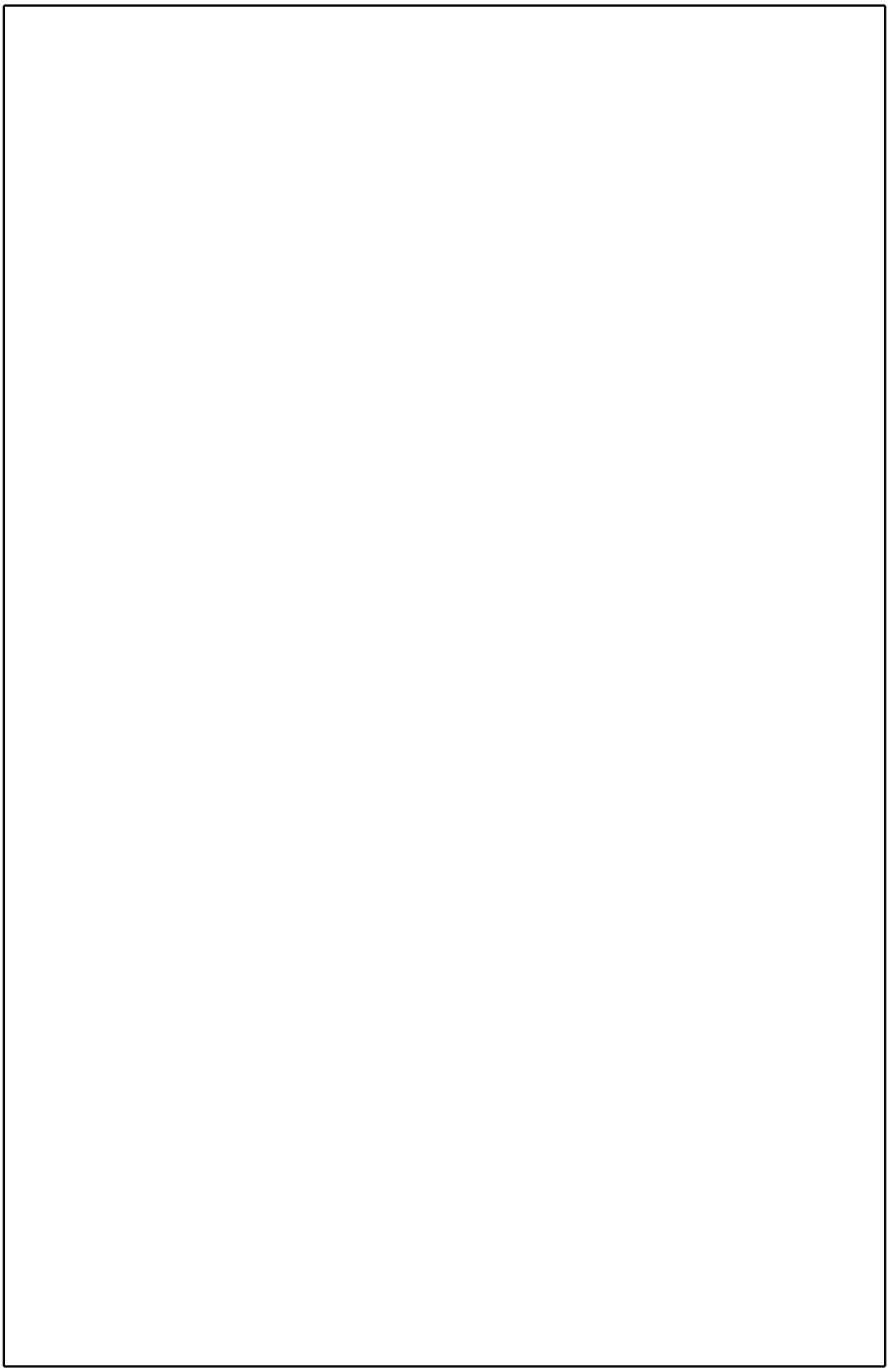
Since the identification, in the early 1990's, that Endothelium-Derived Relaxing Factor (EDRF) was in fact nitric oxide, a significant number of studies have been focused on unraveling the complex chemistry, biology and therapeutic applications of NO. Direct real-time measurement and quantification of NO in various biological matrices is therefore very desirable. Although various methods have been employed to monitor NO in tissues and solutions, it is now generally accepted that electrochemical detection of NO using NO-specific electrodes is the most reliable and sensitive technique available. In the present work, we describe the performance characteristics of several new NO sensors developed recently, including a unique NO StealthSensor®, which has a tip diameter of just 100 nm.



ADDENDUM

Reactive nitrogen species mediate early LDL modifications: A mechanism by which phytoestrogens inhibit LDL oxidation

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It is widely held that oxidative modification of LDL contributes to atherosclerosis, however, the origin of oxidized LDL *in vivo* remains unclear. LDL modification may occur in the vessel wall but this modified LDL is not likely to transit back into the bloodstream. Nevertheless, vascular cells oxidatively modify LDL through a variety of oxidative mechanisms. Since a minimally modified LDL (termed LDL-) is found in plasma and characterized on the basis of its electronegativity, it was used to measure cell-mediated LDL oxidation. LDL- formation by J774 cells took place in transition metal-deficient medium, however, far greater amounts were produced in media containing low μM concentrations of iron and copper. In the presence of metals, up to 70% of the LDL was converted to LDL- over 24 hours. The time-dependent accumulation of LDL- was accompanied by increased lipid peroxidation and protein oxidation as measured by apoB-100 carbonyl content. In the absence of metals, carbonyl formation was negligible and LDL- production was < 5% of total LDL. LDL- formation was also accompanied by nitration of apoB as measured by Western blot analysis of nitrotyrosine content (nY). nY formation was localized to specific regions of apoB and was not metal dependent. Thus, only specific domains of apoB contained nY similar to the nitration found using low concentrations of peroxynitrite. The extent of LDL- production paralleled the levels of apoB-nY, and inhibition of nitration delayed and reduced the amounts of LDL- formed. LDL- and nY formation were inhibited by L-NAME and by estrogenic compounds. The isoflavones Genistein and Equol were

strongly inhibitory at 0.5 μM. Our findings suggest that cell-derived reactive nitrogen species are responsible for early modifications to LDL. This mechanism for cell-mediated LDL oxidation is prevented by estrogen and phytoestrogens, possibly through the inhibition of nitric oxide formation and derived reactive nitrogen species.

Overview of redox signaling: TRX-dependent redox regulation

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Since early 90's, we have characterized mammalian thioredoxin (TRX-1) with redox-active dithiol in the active site as one of the component of redox signaling with multiple functions in cellular processes such as proliferation, apoptosis and gene expression. TRX-1 is induced by a variety of stresses including viral infection (*Annu. Rev. Immunol*. 15, 351-369, 1997, *Current Trends in Immunology* 1, 133-140, 1998). TRX-1 is secreted from the activated cells such as HTLV-I transformed T-cells as a redox-sensitive cytokine with cytokine-like and chemokine-like activities. The promoter of TRX-1 gene contains a series of stress-responsive elements except for HSE. TRX promotes DNA binding of transcription factors such as NF-kB, AP-1 and p53 (*Proc. Natl. Acad. Sci. U. S. A.* 94, 3633-3638, 1997; *J. Biol. Chem.* 274, 35809-3581 5, 1999). Thioredoxin binding protein-2 (TBP-2), which was identical to vitamin D3 up-regulated protein 1 (VDUP1) (*J. Biol.Chemistry* 274:21645-21650, 1999), suppresses the function of TRX and plays an important redox regulatory role in cellular processes, including differentiation of myeloid/macrophage lineage. Potential action of TBP-2/VDUP1 as a redox-sensitive tumor suppressor will be discussed. We will also discuss our recent data on the anti-apoptotic activity of mitochondria-specific thioredoxin-2 (TRX-2) based on in vitro knock-out system (coop with G. Spyrou) as well as a new ER-specific TRX family protein (Y. Matsuo et al. *J. Biol. Chemistry* 2001 in press) of biological functions.

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