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BOOK OF ABSTRACTS

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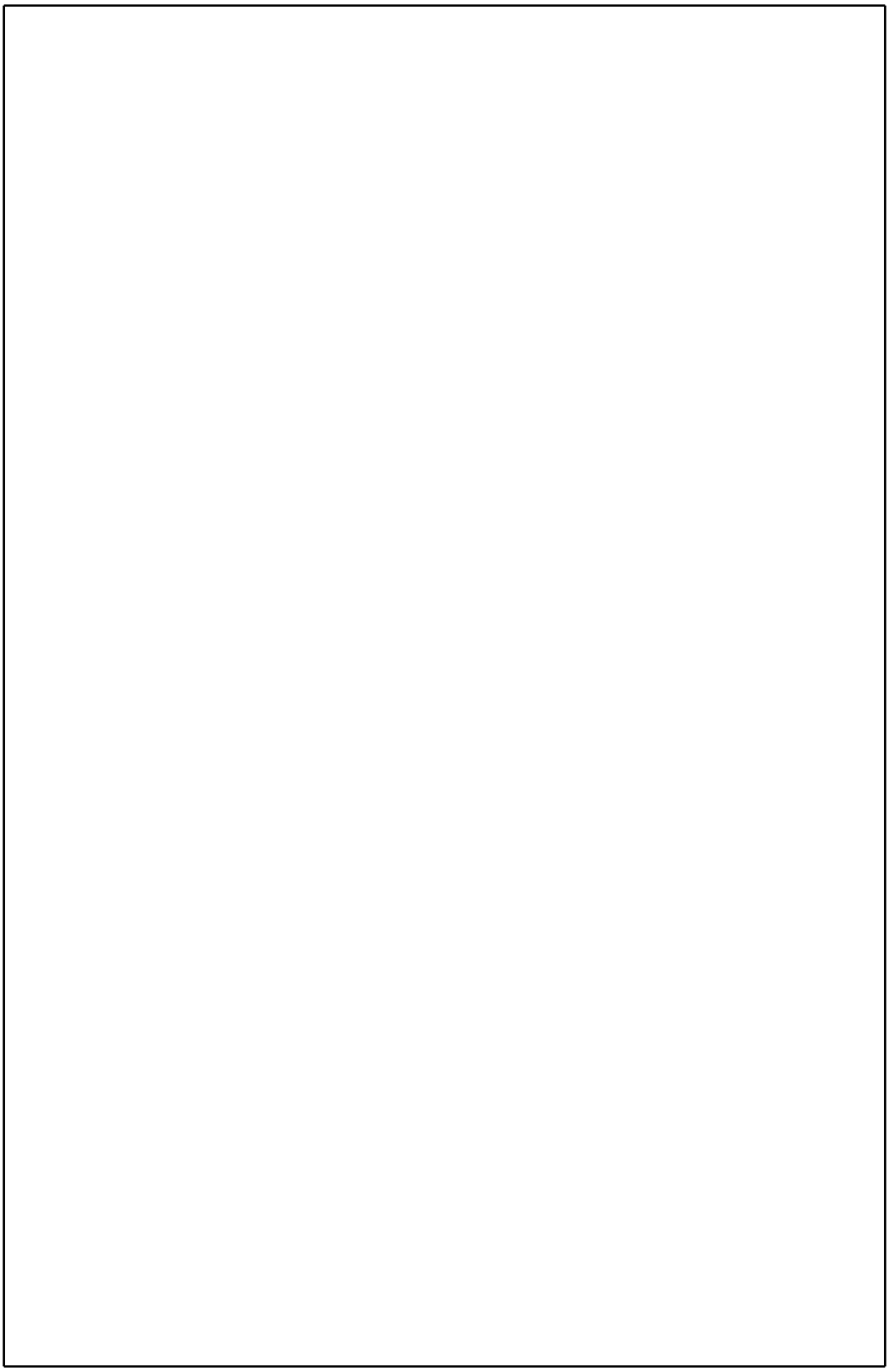

KEYNOTE LECTURE

Abnormal proteins and hydrogen peroxide: Major cytotoxic factors to neurones?

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Accumulation and aggregation of abnormal proteins is a common feature of the major neurodegenerative diseases. Sometimes, mutated proteins are present (α– synuclein or parkin in PD and CuZnSOD in ALS being examples) but more usually the proteins are "normal" but have become oxidized, modified with aldehydes such as HNE and/or nitrated. We have found that mutant proteins associated with PD are not themselves cytotoxic, but render cells in culture more sensitive to damage by several other toxins, including complex I inhibitors and reactive oxygen species such as H_2O_2. Inhibition of the proteasome by chemical inhibitors can mimic many of the effects seen in neurodegenerative disease, including elevated oxidative damage, protein nitration, depletion of GSH, formation of aggregates containing a range of proteins, and apoptosis. Nitration may play a key role in protein aggregation. Thus, proteasomal impairment may be a common feature of neurodegenerative diseases, and agents that upregulate proteasome function might be neuroprotective. Data with Bcl-2 over-expression that illustrate this concept will be presented. Another issue recently addressed is the question of the mechanism of toxicity of L-DOPA and dopamine to neurons in culture; the results obtained cast serious doubts on the validity of much cell culture work.

SESSION I
NITRIC OXIDE

Few radicals from the adduct between $ONOO^-$ and CO_2

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CO_2 catalyses the isomerisation of $O{=}NOO^-$ to NO_3^- *via* an intermediate, presumably $O{=}NOOCO_2^-$, which has an absorption maximum at $\lambda > 600$ nm.[1] The reflection spectrum of solid $N(CH_3)_4ONOO$ exposed to CO_2 shows a similar band near 650 nm; this absorption decays over minutes and shows only a very small ESR signal that probably is due to the presence of trace amounts of superoxide.

We found by ESR that reaction of $O{=}NOO^-$ with CO_2 forms some $CO_3^{\cdot -}$ and $NO_2^{\cdot}$ radicals *via* homolysis of the O-O bond in $ONOOCO^{2-}$.[1] We recently determined the extent of radical formation by mixing $O{=}NOO^-$, CO_2 and $NO^{\cdot}$. The latter reacts with $CO_3^{\cdot -}$ and $NO_2^{\cdot}$ radicals to form, effectively, 3 NO_2^- *per* homolysis; $ONOOCO^{2-}$ that does not undergo homolysis yields NO_3^- and CO_2. Based on the NO_3^- and NO_2^- analyses after removal of $NO^{\cdot}$, the extent of conversion to NO_3^- is (96 ± 1)% and that of homolysis (3 ± 1)%, respectively;[2] the extent of homolysis is significantly less than the *ca.* 30% reported in the literature.

Stopped-flow experiments in which CO_2 solutions were mixed with alkaline $ONOO^-$ solutions indicate the formation of at least one intermediate: The initial absorption at 302 nm is less than that of $ONOO^-$ which indicates that reactions take place within the mixing time, and this absorption is dependent (but not linearly) on the $ONOO^-$ and the CO_2 concentration.

Supported by the ETH and the Swiss Nationalforschung.

1. Meli, R., Nauser, T., & Koppenol, W.H. (1999) Helv. Chim. Acta **82**:722-725.

2. Meli, R., Nauser, T., Latal, P. & Koppenol, W.H. (2002) J. Biol. Inorg. Chem. **7**:31-36.

Quantitation with fluorescent particles of reactive oxidants produced by phagocytes

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In earlier studies [Jiang et al., *Chem. Res. Toxicol.* (1997) **10**, 1080-1089; Jiang & Hurst, *J. Biol. Chem.* (1997) **272**, 32767-3277 2], we demonstrated the utility of fluorescein-conjugated μm-sized particles for investigating intraphagosomal reactions in neutrophils. A unique feature of these particles is that the dye is covalently attached to the beads through a cystamine linker group; consequently, it can be released by disulfide exchange with added sulfhydryl compounds and subsequently recovered for chemical analyses in near-quantitative yield from cellular environments. Although unopsonized beads triggered no phagocytic response, they were avidly phagocytosed by human neutrophils when opsonized with serum. By measuring the fluorescence changes accompanying phagocytosis and the extent of ring chlorination of the dye, we established that microbicidal amounts of HOCl were generated in myeloperoxidase (MPO)-catalyzed reactions within the phagosome during the respiratory burst. Because fluorescein is a phenolic compound, it also undergoes MPO-catalyzed nitration in the presence of NO_2^-. At physiological levels of Cl^-, when $[NO_2^-] \geq 1$ mM neutrophil, stimulation caused both chlorination and nitration of the particle-bound dye in extracellular environments. However, no fluorescein nitration was detected on phagocytosed particles at any NO_2^- concentration, an effect potentially attributable to the limited intraphagosomal volume.

Recent interest in macrophage generation of reactive nitrogen species has provoked us to undertake parallel studies with isolated rat peritoneal macrophages and RAW 264.7 cells, an immortalized macrophage-like cell line. Preliminary results indicate that probe

nitration does indeed occur, but not until NO_2^- has accumulated in the medium. This nitration reaction can apparently take place well after cessation of nitric oxide biosynthesis, provisionally excluding peroxynitrite and N_2O_3 as nitrating agents. The local distribution of oxidant within the cellular suspensions is being investigated using a new particulate probe comprising 2,7-dichlorodihydrofluor escein coordinated to 1-μm latex particles. Results of these ongoing studies will be presented.

The complex roles of nitric oxide in airway inflammation

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The production of nitric oxide (NO·) is generally increased during inflammatory diseases of the respiratory tract, due to induction of inducible nitric oxide synthase (NOS2) in epithelial or inflammatory cells, but the overall role of NO· in such inflammatory conditions is still largely unclear. Although NO· has anti-inflammatory properties, oxidant-producing systems that are activated during inflammation enhance metabolism of NO· to more reactive nitrogen species (RNS) that can contribute to inflammation. We have used various approaches in studies with airway epithelial cells or NOS2 knockout animals, to investigate the involvement of NO· or NOS2 on inflammatory processes in the airways. In one set of *in vivo* experiments, we exposed wild-type and NOS2-deficient mice to 1 ppm O_3 (8 hr/day for 3 days), and monitored airway inflammation and lung injury. Analysis of bronchoalveolar lavage (BAL) fluids showed dramatically more neutrophil influx in NOS2-deficient mice than in wild-type mice, which was associated with dramatic increases in BAL protein, macrophage inflammatory protein (MIP)-2, and MMP-9 (largely originating from neutrophils). These results implicate NOS2-derived NO· in inflammation, possibly through enhanced formation of RNS. Indeed, exposure to O_3 also resulted in increased protein nitrotyrosine levels in lung tissue, in both wild-type and NOS2-deficient mice, suggesting that protein nitration occurs independent of NOS2 induction and associates more closely with inflammation. In another set of experiments, acute lung inflammation was induced in mice by intranasal instillation of 300 µg/kg lipopolysaccharide (LPS), which was found to result in dramatic neutrophil efflux into the airspaces, peaking after 24 hrs ($1.02 \pm 0.23 \times 10^6$ BAL neutrophils) and then resolving after 3 days. This acute inflammatory response was significantly reduced in NOS2-deficient mice ($0.41 \pm 0.19 \times 10^6$ neutrophils after 24 hrs), and LPS-induced increases in BAL protein and

BAL MMP-9 levels were also slightly lower in NOS2-deficient mice compared to wild-type mice. Nitrotyrosine analysis showed no significant differences between wild-type and NOS2-deficient mice. Interestingly, lung tissue MMP-9 gene expression was induced in LPS-treated mice, and the increase in MMP-9 mRNA appeared to be somewhat higher in NOS2 deficient animals. This latter finding suggests enhanced activation of nuclear factor (NF)-κB (which is essential for epithelial MMP-9 expression) in the absence of NOS2, which would be consistent with proposed inhibitory effects of NO· on NF-κB activation. Studies with human bronchial epithelial cells showed that inhibitory effects of NO· on tumor necrosis factor-induced NF-κB activation and MMP-9 expression are due to *S*-nitrosation and not to NO· itself. Collectively, the overall role of NOS2 in the regulation of airway inflammation is quite variable, and both pro- and anti-inflammatory effects can be observed, depending on the model used.

SESSION II
NITRIC OXIDE

Nox1: A redox-based regulatory switch for inducible nitric oxide synthase

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Nitric oxide (NO) derived from the inducible form of NO synthase (iNOS) plays dichotomous roles in modulating inflammatory responses. Thus, for NO to serve as a versatile signaling molecule, its biosynthesis must be under exquisite control. Whereas numerous proinflammatory mediators have been identified which stimulate the expression of iNOS, mechanisms leading to the repression of this gene during the resolution phase of inflammation have not been characterized. Herein, evidence is provided that a homolog of the phagocyte NADPH oxidase, Nox1, serves as a growth factor-responsive and redox-based repressor of iNOS expression in non-phagocytic cells. We have observed that cytokine-inducible expression of iNOS is repressed by pretreatment of smooth muscle cells with growth factors (PDGF and Ang-II), and that this inhibitory effect is dependent upon hydrogen peroxide (H_2O_2) production from Nox1. Additionally, cells pretreated with the H_2O_2-producing agent DMNQ, were incapable of upregulating iNOS in response to cytokines. Supporting this notion, NIH 3T3 cells stably transfected with Nox1 did not express iNOS nor produce NO in response to cytokines, but did when co-transfected with catalase. These observations provide evidence that low levels of H_2O_2, produced by Nox1, can provide cells with a means of regulating the expression of iNOS and modulating the progression of inflammatory responses.

Effects of gender on reduced-size liver ischemia and reperfusion injury in mice: Role of nitric oxide

HIROHISA HARADA, KEVIN P. PAVLICK, IAN N. HINES, JASON M. HOFFMAN, LAURA GRAY, AND MATTHEW B. GRISHAM

Hepatic resection with concomitant periods of ischemia and reperfusion (I/R) is associated with liver resectional surgery as well as reduced-size liver (RSL) transplantation (e.g. living donor or split liver transplantation). Unfortunately, the I/R induced in the liver by these surgical manipulations may impair liver regeneration ultimately leading to liver failure. Data obtained from both clinical and experimental studies suggest that gender may influence the outcome following such types of procedures. In addition, recent studies from our laboratory suggest that endothelial cell nitric oxide synthase (eNOS)-derived nitric oxide (NO) may play an important role in modulating full size liver I/R-induced injury. Therefore, we wished to assess the effects of gender in a mouse model of RSL+I/R injury as well as determine the role that 17β-estradiol (E2) plays in eNOS expression and liver injury in this model. Anesthetized female and male wild type (wt) mice genetically deficient in eNOS gene (eNOS-/-) were subjected to 70% liver ischemia for 45 min followed by resection of the remaining 30% non-ischemic lobes. Liver injury was assessed by quantifying serum ALT levels whereas the message levels of eNOS in the liver tissue were evaluated using RT-PCR. We found that liver injury was significantly greater in males than in females at 20 h following surgery. Furthermore, we observed that 100% of the female mice survived indefinitely post surgery whereas all male mice died within 5 days following RSL+I/R. Administration of E2 to **male** mice 24 h prior to RSL+I/R followed by a second injection 24 h later resulted in significant reduction in serum ALT levels at 20 h following RSL+I/R and a dramatic improvement in the 7-day survival rate (88% vs 0% for

E2-treated vs control). Treatment of **female** mice with the selective E2 receptor antagonist ICI 182,780 resulted in enhanced liver injury as assessed by elevations of serum ALT levels at 20 h following RSL+I/R and decreased 7-day survival rates (25% vs 86% for ICI 182,780-treated vs control females, respectively). We also found that eNOS message levels were increased in females (11-fold over males) and E2-treated males (14-fold over control males) at 1 h post-surgery. Furthermore, treatment of females with the E2 receptor antagonist resulted in a 65% reduction of eNOS message levels at 1 h post-surgery compared to their vehicle-treated controls. Interestingly, iNOS message was not detected in the liver at any time-point in any group. Finally, we found that eNOS-/- female mice were much more sensitive to the damaging effects of RSL+I/R compared to wt females as assessed by increased serum ALT levels. In fact, all eNOS-/- female mice died within 2 days following RSL+I/R. We conclude that the protective effect afforded to female mice or male mice treated with E2 in this model of RSL+I/R appears to be due to the E2-dependent upregulation of eNOS-derived NO. We propose that E2 agonists and/or NO donors may prove useful in protecting the liver from RSL+I/R injury.

S-Nitrosothiol biochemistry and the normal response to hypoxia

BENJAMIN GASTON

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The effects of hypoxia on ventilation and protein expression *in vivo* have long been appreciated, but the signaling mechanisms underlying these responses remain poorly understood. Here, we define a novel pathway in which increased minute ventilation and gene transcription are signaled by deoxyhemoglobin- derived S-nitrosothiols. Specifically, we demonstrate that 1) S-nitrosocysteinyl glycine and S-nitroso-L-cysteine — but not S-nitroso-D-cysteine — reproduce both the ventilatory effects of hypoxia at the level of the nucleus tractus solitarius; 2) plasma from deoxygenated blood — but not from oxygenated blood — produces the ventilatory effect of both S-nitrosothiols and hypoxia; 3) this activity is mediated by S-nitrosoglutathione (GSNO); 4) GSNO also upregulates hypoxia-associated gene expression in normoxia by stabilizing the a subunit of hypoxia inducible factor 1 through inhibition of its ubiquitination; and 5) GSNO activation by γ-glutamyl transpeptidase (γGT) is required for its ventilatory and gene-regulatory effects. Further, we show that the normal response to hypoxia is impaired in a knockout mouse lacking γGT. These observations suggest that S-nitrosothiol biochemistry is of central importance to the normal response to hypoxia.

Regulation of apoptosis by cytochrome *c* nitrosylation

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Cytochrome *c* plays a critical role in many apoptotic pathways. When mitochondria receive an apoptotic signal, cytochrome *c* is released from the mitochondrial intermembrane space into the cytoplasm. Cytoplasmic cytochrome *c* forms a complex with Apaf-1 and caspase-9 leading to the activation of downstream caspases and subsequent apoptotic cell death. The mechanisms regulating cytochrome *c* function during apoptosis are poorly understood. Our preliminary data indicates that cytochrome *c* released into the cytoplasm one hour after Fas stimulation is nitrosylated. When cytochrome *c* release into the cytoplasm is inhibited by Bcl-2 or Bcl-XL overexpression, nitrosylated cytochrome c is found predominantly in the mitochondria. This data suggests that during Fas-induced apoptosis, cytochrome *c* is nitrosylated in mitochondria and then rapidly released into the cytoplasm. Finally, we demonstrate that nitrosylation of cytochrome *c* increases caspase-3 activation. This is the first demonstration of a posttranslational modification regulating cytochrome *c* function during apoptosis. The data suggest that cytochrome *c* nitrosylation is a novel biochemical mechanism underlying some of the pro-apoptotic effects of nitric oxide.

SESSION III
THE ANTIOXIDANT VITAMINS C AND E

Vitamin E kinetics in pregnancy and lactation 2:1 Preference for *RRR*- compared with *all rac*-α-tocopherols

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There is a dirth of information available concerning vitamin E delivery to the fetus during pregnancy or to infant tissues during lactation. The objective of the present investigation was to compare the relative abilities of synthetic vitamin E (*all-rac*-α-tocopherol, contains 8 different stereoisomers) with *RRR*-α-tocopherol, which occurs naturally, to enrich piglet tissues when fed as α-tocopheryl acetates to sows during pregnancy and lactation. α-Tocopherol delivery to fetuses and to suckling piglets was monitored by feeding 150 mg each of d3-*RRR*-α– and d6-*all-rac*-α-tocopheryl acetates to 3 pregnant sows daily from 7 d before to 7 d after giving birth. Labeled and unlabeled vitamin E concentrations were measured in sow plasma and milk, and in piglet (n=9) plasma and tissues at birth, 7 and 21 d. At birth, prior to suckling, despite elevated sow plasma deuterated α-tocopherol concentrations, no labeled α-tocopherol was detected in piglet plasma or tissues. Sow plasma and milk d3-α- to d6-α-tocopherol concentrations were 2:1, leading to a 2:1 ratio in suckling piglet plasma and tissues. In piglets at d 7 compared to birth, most tissues contained a 10-fold increase in total α–tocopherol. In conclusion, pigs discriminate between *RRR*- and *all-rac*-α-tocopherols with a 2:1 preference for *RRR*-α-tocopherol, well above the USP bioequivalence ratio of 1.36:1 *RRR*- to *all-rac*-α–tocopherol. Following initiation of suckling, piglets' plasma and tissues demonstrated a dramatic, 10-fold increase in vitamin E concentrations, emphasizing the limited placental vitamin E transfer and the importance of milk for enhancing the vitamin E status of the newborn.

Mechanisms of vitamin E metabolism

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The side chains of tocopherols are degraded by ω- and subsequent ß-oxidation. The ß-oxidation steps have been proven by the identification of the final products, carboxyethyl hydroxychromans (CEHC), their precursers, carboxymethylbutyl hydroxychromans (CMBHC), and the precurser of α-CMBHC, α-carboxymethylhexyl hydroxychroman (α-CMHHC). The ω-oxidation is less well understood. It has been deduced from the inhibition of γ-CEHC production from γ-tocopherol by ketoconazole and sesamine, inhibitors of CYP3A4, a member of the cytochrome P450 family, and from an increase in *all rac* α-tocopherol-derived α-CEHC-release by rifampicin-treated HepG2 cells. This pathway may be common for all tocopherols and also tocotrienols since α-and γ-CEHC have been shown to be endproducts of α-and γ-tocotrienol metabolism. Metabolic rates, however, are highly different for different forms of vitamin E. We further investigated the degradation of tocopherols and tocotrienols. Incubation of HepG2 cells with *all rac* α- and γ-tocopherol resulted in an excretion of CEHCs, CMBHCs and CMHHCs. The amount of accumulated metabolites of γ-tocopherol was up to 100 times higher than that of metabolites of α-tocopherol. Tocotrienols were degraded to high amounts of CEHCs, CMBHCs and to their immediate precurser CMH(en)HCs. Estimation of the relative relevance of side chain degradation of tocopherols and tocotrienols has, thus, to consider the production of all metabolites and not only the final product, CEHC.

γ-Tocopherol metabolism and its relationship with α-tocopherol in humans

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Eighty years after the discovery of vitamin E there is still incomplete understanding of its function and metabolism in humans. This is particularly true in the case of γ-tocopherol (γ-T), which, due to its lower plasma concentration and bioactivity in animal bioassays, has until recently been considered to be of minor importance compared to α-T. The emergence of new evidence of possible health benefits of γ-T has led some investigators to question this conclusion. For example, low γ-T concentrations and a high α- to γ-T ratio have been reported in patients with CHD compared to controls (Ohrvall et al, 1996; Kontush et al. 1999) and in a population with a high incidence of CHD (Kristenson et al, 1997). In addition, information has appeared which suggests a possible role for γ-T and its main catabolite 2,7,8-trimethyl-2-(b-carboxyethyl)-6-hydroxychroman (γ-CEHC) in both defense against nitrogen oxide species formed during the activation of inflammatory cells. Moreover, γ-CEHC has been reported to play a role in the regulation of natriuresis (Wechter et al, 1996), while the equivalent α-T metabolite has no such activity.

Given these findings, more attention is being paid to the function and metabolism of the non-α-T vitamers. With this aim in mind we undertook some work using deuterium labeled γ-T in man. Specifically we wanted to understand the early events in the metabolism of γ-T and to gain a better appreciation of its interactions with α-T.

We administered 100 mg of deuterium labeled (d2)-γ-T in a single dose with a standard meal to 21 healthy subjects (age 32. 6±8.3 SD). The concentrations of α- and γ-T in plasma, as well as the excretion of their metabolites in first morning void urine were evaluated by HPLC and GC-MS analysis at day 0 (pre-) and 1, 3,

7 and 10 days post-supplementation. In a few subjects, blood and urine samples were also collected at 6 , 9 and 12 hours post-supplementation to look at very early events.

In all subjects we observed a transient rise in plasma γ-T concentration, which resolved with 72 hours. Neither plasma α-T nor retinol levels were affected by the γ-T supplementation. The urinary excretion of γ-CEHC followed a similar time pattern to that of plasma γ-T, with the majority of the increase in metabolite excretion being present as d2-γT form. Urinary d0-γ-CEHC levels were only marginally increased.

This stable isotope study illustrates the rapid metabolism and loss of ingested γ-T in humans. Urinary γ-CEHC appears to represent a main, but not exclusive, route for excretion of γ-T metabolites. Furthermore, it is of interest to note that the concentration of α-T in plasma and its metabolism, are not influenced by γ-T supplementation.

Ohrvall et al, J Intern Med. 239(2):111-7,1996
Kontush et al. Atherosclerosis.;144(1):117-22.1999
Kristenson et al, BMJ; 314(7081):629-33. 1997.
Wechter et al, Proc Natl Acad Sci USA 93: 6002-7, 1996

Bioavailability and biopotency of Vitamin E in humans: An ongoing controversy

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Bioavailability is defined as the rate and extent at which an active compound appears in blood. Potency is a measure of effects of an active compound or moiety, based on clinical or biochemical endpoints. Regarding the potency assessment of vitamin E forms, such endpoints exist in animal species, *e.g.*, myopathy and fetal resorption in rats. In contrast, in humans, suitable clinical endpoints are lacking. The potencies of tocopherol and tocotrienol vitamers, including the most highly active α-tocopherol from natural sources (one compound, RRR) and from chemical synthesis (an *all-rac*emic mixture of 8 enantiomers including RRR) were determined by the rat fetal resorption assay. The latter yielded a ratio of potency for *all-rac* : RRR of 1 : 1.36 (USP, 1979). This ratio has been disputed for humans. Since the sixties, human studies were carried out that compared RRR and *all-rac* in parallel groups. Some found ratios of bioavailability citeria for RRR : *all-rac* higher than 1.36 : 1 resulting in the claim that this reflects a higher ratio in potency. This is not valid because bioavailability reflects potency only if chemically identical compounds, and hence, identical kinetics, are compared. In the nineties, human studies were done with equimolar doses of d3-RRR and d6-*all-rac* given simultaneously. The studies provided new insights into the regulation of plasma vitamin E concentration, from absorption to renal CEHC excretion. Particularly, the pivotal role of hepatic α-tocopherol transfer protein (α-TTP) in regulating the biodiscrimination of homologs and stereoisomers was identified. It is believed that the slower plasma elimination kinetics of RRR compared to the SRR-enantio mer (one of the 8 enantiomers in *all-rac*) is due to the higher affinity of α-TTP for RRR. Recently, in defining new RDA values, the Food and Nutrition Board has proposed that only RRR and the

three other 2R-configurated stereoisomers of *all-rac* exhibit vitamin E activity, but not the non-α-tocols and tocotrienols. This is at variance with the present state of knowledge in human and animal nutrition. It is argued that β-, γ- and δ-tocopherols and the tocotrienols are not converted to α-tocopherol and are recognized poorly by α-TTP. Peroxide-induced hemolysis *ex vivo* that reflects antioxidant activity was used as the biochemical parameter. Vitamin E effects on endothelial and platelet functions that are independent of antioxidant activity have not yet been utilized as functional tools for establishing relative potencies. In view of the growing awareness of the potential importance of vitamin E for prevention of age-related diseases, studies are needed that use suitable and sensitive endpoints for delineating vitamin E effects and for establishing relative potencies of RRR and *all-rac* and also for γ-TOH, the predominant tocopherol in the US diet.

Vitamin C pharmacokinetics in healthy humans

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Recommendations for optimum vitamin C intake can be based in part on relationships between dose ingested and resulting concentrations. This information was obtained from 22 healthy human subjects, 7 men and 15 women ages 19-27, who were inpatients in a 6 month depletion- repletion study. For the entire study subjects consumed a vitamin C diet providing < 5 mg daily of vitamin C and sufficient for all other nutrients. Subjects consuming the diet were depleted of vitamin C (plasma < 8 uM), and then received escalating vitamin C doses, from 30 to 2500 mg daily. Steady state was achieved for each dose before the dose was increased. Vitamin C was measured in plasma, neutrophils, lymphocytes, monocytes, platelets, and urine; bioavailability measurements for each dose were performed at steady state over 36 hours; and samples were obtained to assay biomarkers of oxidative stress. For both men and women there was a steep sigmoid relationship between dose and plasma concentration for doses between 30 and 100 mg daily, but the curve for women was shifted to the left compared to men ($p=0.01$). The first dose beyond the steep portion of the sigmoid curve was 200 mg daily. Cells were saturated at 100-200 mg daily. Plasma was saturated at 400 mg daily, at concentrations of 75-80 uM. Dehydroascorbic acid was not detected in plasma. The threshold dose for urine excretion was between 60 and 100 mg daily, and the entire absorbed amount was excreted for doses of 500 mg and higher. F2-Isoprostanes were unchanged by vitamin C in women at all doses. These data indicate that vitamin C concentrations are tightly controlled in humans as a function of dose. Data for plasma and tissue saturation, transporter kinetics, bioavailability, and urine excretion are consistent with observational data indicating benefit from 5 varied daily servings of fruits and vegetables, providing approximately 200 mg daily.

Vitamin C: Transport and new biology

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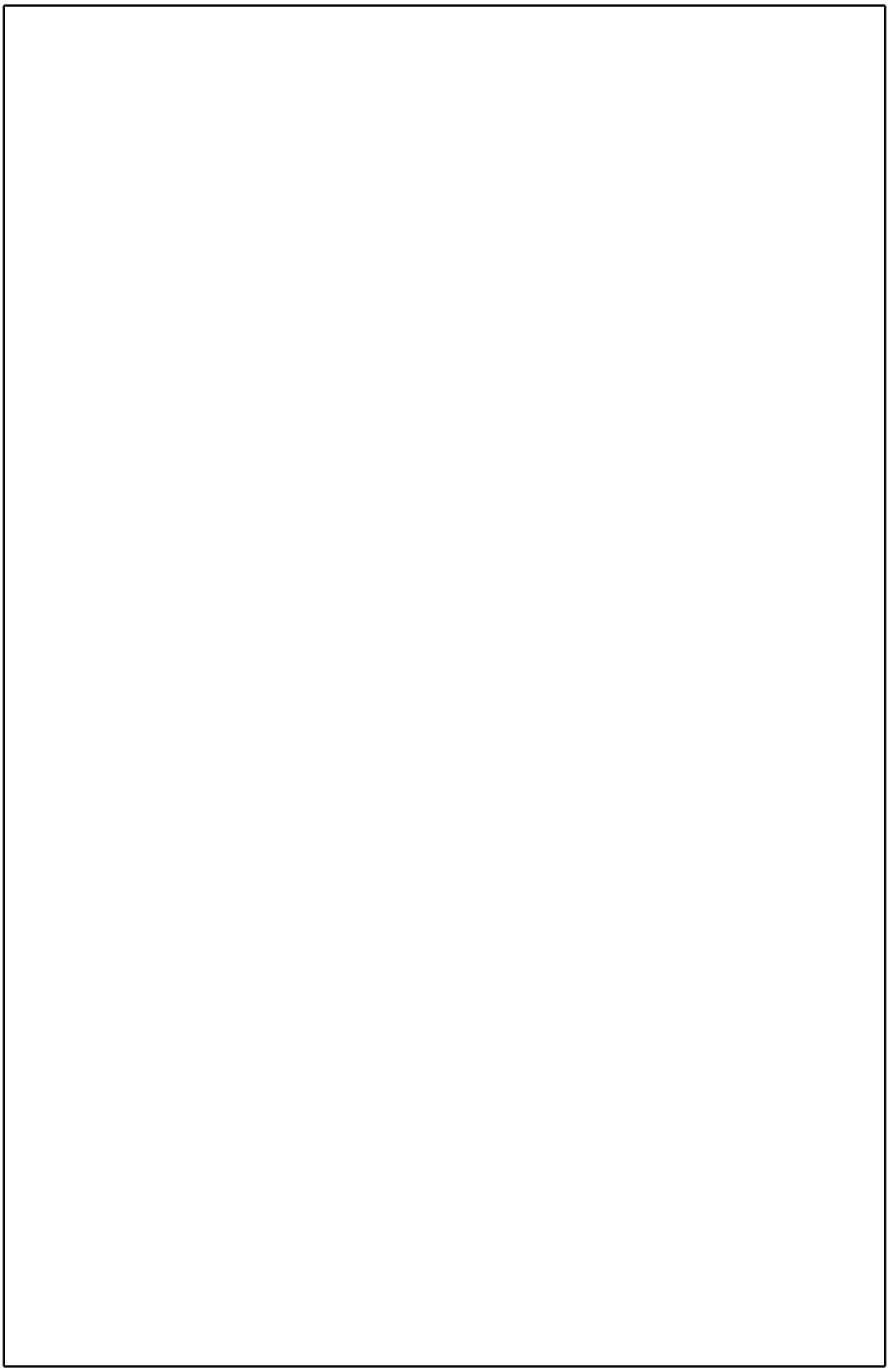
In 1993 we reported on a universal mechanism for cellular uptake of vitamin C in the form of dehydroascorbic acid (DHA) (Nature: 364; 79-82, 1993). Vitamin C circulates in the plasma as ascorbic acid and reaches the extracellular milieu in that form. Outside the cell it is oxidized to DHA, and transported as DHA into cells via the facilitative glucose transporters (GLUTs). Once inside the cell, DHA is rapidly reduced to ascorbic acid. In this way some cells are able to achieve intracellular vitamin C concentrations almost two orders of magnitude greater than the ambient ascorbic acid concentration. This universal mechanism of transport also implies a homeostatic mechanism whereby extracellular oxidative events lead to increased concentrations of vitamin C intracellularly. Some cancer cells use this mechanism to obtain vitamin C, relying on stromal cells to oxidize extracellular ascorbic acid. Specialized cells can transport ascorbic acid directly through sodium dependent ascorbate co-transporters, which may also be important for intracellular compartment transfer of ascorbic acid. Inside the cell ascorbic acid acts as a strong antioxidant and a co-factor for at least 8 enzymes, many involved in collagen synthesis. Appreciation of the role of redox in cellular signaling pathways has pointed to effects of antioxidants on cell signaling and resultant biologic activities. We analyzed the effect of vitamin C on GM-CSF signaling. We found that loading cells with high concentrations of vitamin C leads to inhibition of GM-CSF signaling with a predominant effect between the receptor and JAK2 kinase activation. We also have data supporting the concept that high intracellular concentrations of vitamin C inhibit oxidative DNA damage. DHA is being developed as a potential therapeutic agent. Collaborative studies with scientists at Columbia University and Progenics, Inc., led to results indicating that DHA ameliorates the effect of experimental stroke in a rodent model. These new concepts regarding the transport and biological activity of vitamin C have broadened our understanding of vitamin C and the potential therapeutic application of antioxidants.



SESSION IV
THE ANTIOXIDANT VITAMINS C AND E

Effect of selenium and vitamin E on differential gene expression *in vitro* and *in vivo*

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A wide spectrum of beneficial activity to human health has been advocated for selenium (Se) and vitamin E (VE). More recently the ability of antioxidants to affect gene expression and cell response has been reported, providing a novel mechanistic perspective on their biological activity. To examine molecular targets of Se and VE, we applied cDNA array technology to define the transcriptional response of these micronutrients in rat liver and in primary cultured human endothelial cells.

In rats VE deficiency alone did not induce any significant changes in expression profile among the genes evaluated (Fischer et al. 2001). However Se deficiency lead to a down-regulation of Se-dependent cGPx and to an induction of genes, encoding for detoxifying enzymes in liver. Combined VE and Se deficiency was characterized by alterations in the expression level of genes encoding for proteins involved in inflammation and acute phase response. Additionally, a significant down-regulation in the expression level of genes important in the inhibition of apoptosis, cell cycle and antioxidant defense was demonstrated.

In primary human endothelial cells (HUVEC) oxidized LDL induced changes in the mRNA levels of genes that encode for transcription factors, cell receptors, adhesion molecules and extracellular matrixproteins. Several genes induced by oxidized LDL were down-regulated by vitamin E.

The experimental approach identified several novel Se and VE sensitive genes *in vitro* and *in vivo*. DNA microarrays might lead to better insights into the molecular mechanisms of antioxidants such as Se and VE, thereby offering a novel strategy in nutrition research.

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Vitamin E in cell signaling

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Although it is common believe that phenolic compounds like vitamin E exert only a protective role against free radical damage, it is also known that antioxidant molecules can exert additional biological functions. The new biochemical face of vitamin E has been first described in 1991, with the inhibitory effect on cell proliferation and protein kinase C activity. Non-antioxidant effects of α-tocopherol (α-T) take place at the level of cell signaling and gene expression. Other antioxidants, and in particular β-tocopherol, do not cause the cell responses described with α-T. When added together, β-tocopherol prevents the effects of α-T that cannot therefore be related to the radical scavenging of this molecule. The data suggest the existence of a ligand/receptor mechanism at the basis of α-T action. Oxidant stress causes diminution of antioxidant molecules, such as α-T. α-T (10-50 microM) specifically increases protein phosphatase 2A1 activity. This activation is followed by PKC-α dephosphorylation and by a decrease of PKC activity. A PKC dependent modulation of gene expression by α-T is observed (CD36, Connective Tissue Growth Factor, collagenase-MMP-I and Tropomyosin). Consequent to the effect on PKC α-T has been shown (in our laboratory and by others) to prevent cell adhesion, to inhibit platelet aggregation, to prevent smooth muscle cells proliferation and to inhibit PKC dependent oxygen burst. Transcriptional regulation by α-T of scavenger receptor CD36 and of Connective Tissue Growth Factor is however not related with an inhibition of PKC. Three new α-T associated proteins (TAPs) from human tissues, have structural motifs common to the CRAL-TRIO

family. Human TAP is ubiquitous, but more highly expressed in liver, prostate and brain tissue. TAP has GTPase activity suggesting regulatory cell functions.

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α-Tocopherol transfer protein (α-TTP) and familial isolated vitamin E deficiency

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Vitamin E (α-tocopherol) is the most potent lipid-soluble antioxidant in biological membrane. Patients with ataxia and isolated vitamin E deficiency (AVED) have low or undetectable plasma vitamin E concentration and exhibit neurological dysfunction and muscular weakness. It is now established that α-tocopherol-transfer protein (α-TTP), a cytosolic liver protein that binds specifically α-tocopherol, is defective in AVED patients, indicating that α-TTP is a major determinant of plasma α-tocopherol level. Recently, we established a mouse model lacking α-TTP by targeted mutagenensis. This animal model for human AVED patients is suitable for examination of the complex pathophysiology of diseases associated with vitamin E deficiency. Male α-TTP(-/-) mice were fertile; however, placentas of pregnant female were severely impaired with marked reduction of labyrinthine trophoblast, and embryo died at mid-gestation even when fertilized eggs of α-TTP (+/+). α-TTP(-/-) mice showed ataxia and retinal degeneration after 1 year of age. The neurological phenotype of α-TTP(-/-) mice was much more severe than that of wild-type mice when maintained on an a-tocopherol-deficient diet. Lipid peroxidation in α-TTP(-/-) mice brains showed a significant increase, especially in degenerating neurons. The use of excess α-tocopherol dietary supplement by α TTP(-/-) mice prevented placental failure, allowed full-term pregnancies, suppressed lipid peroxidation, and almost completely prevented the development of neurological symptoms.

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Vitamin C-induced decomposition of lipid hydroperoxides to endogenous genotoxins

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Epidemiological data suggest that dietary antioxidants play a protective role against cancer. This has led to the proposal that dietary supplementation with antioxidants such as vitamin C may be useful in disease prevention. Unfortunately, vitamin C has proved to be ineffective in cancer chemoprevention studies. Furthermore, concerns have been raised over potentially deleterious transition metal ion-mediated pro-oxidant effects. We have now discovered that vitamin C induces lipid hydroperoxide decomposition to the DNA-reactive bifunctional electrophiles 4-oxo-2-nonenal, 4,5-epoxy-2(*E*)-decenal, and 4-hydroxy-2-nonenal. The 4,5-epoxy-2(*E*)-decenal is a precursor of etheno-2'-deoxyadenosine, a highly mutagenic lesion found in human DNA. Vitamin C-mediated formation of genotoxins from lipid hydroperoxides in the absence of transition metal ions could help explain its lack of efficacy as a cancer chemoprevention agent.

Vitamin C does not act as a pro-oxidant towards lipid and proteins in human plasma exposed to the complete Udenfriend system

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The combination of ascorbate, transition metal ions and hydrogen peroxide (H_2O_2) forms an efficient hydroxyl radical generating system called "the complete Udenfriend system." Although the pro-oxidant role of ascorbate in this system has been well characterized *in vitro*, it is uncertain whether ascorbate acts as a pro-oxidant under physiologically relevant conditions. To address this question, freshly obtained human plasma was depleted of endogenous ascorbate with ascorbate oxidase, or supplemented with increasing concentrations of ascorbate. Subsequently, transition metal ions and/or H_2O_2 were added, and plasma antioxidants and lipid and protein oxidation products were measured. We found that ascorbate (46 - 300 μM) was rapidly depleted in the presence of iron (50 or 100 μM) or copper (30 or 60 μM). In contrast, the rate of plasma α-tocopherol consumption was not affected by either the addition of iron, ascorbate or H_2O_2 (200 μM). Despite the fact that iron and copper interacted with ascorbate, causing rapid oxidation of the latter, formation of lipid hydroperoxides did not occur in plasma containing ascorbate. Furthermore, even when H_2O_2 was added in addition to ascorbate and metal ions (the complete Udenfriend system), ascorbate protected against iron- and copper-induced lipid peroxidation. Conversely, the rate of lipid peroxidation and the amounts of lipid hydroperoxides formed were highest in plasma devoid of ascorbate. Ascorbate also did not enhance metal-dependent oxidation of plasma proteins, as measured by protein carbonyl formation. These results are consistent with our previous observations of i) antioxidant effects of ascorbate in human plasma exposed to iron alone (incomplete Udenfriend sys-

tem); ii) lack of a correlation between non-protein bound, bleomycin-detectable iron and lipid and protein oxidation products in plasma of premature infants; and iii) reduced F2-isoprostane levels in iron-loaded guinea pigs fed high as compared to low doses of vitamin C. Taken together, our data indicate that even in the presence of high concentrations of transition metal ions and H_2O_2, ascorbate acts as an antioxidant that inhibits, and does not promote, lipid and protein oxidation in human plasma and *in vivo*.

Vitamin C and endothelial nitric oxide synthesis

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Reduced generation of endothelium-derived nitric oxide (NO) leads to vasomotor dysfunction and disordered thromboregulation and has been implicated in a number of vascular diseases. The present study investigates if vitamin C which has been shown to reverse impaired endothelium-dependent vasodilation in patients with atherosclerosis affects cellular NO synthesis. Pretreatment of human endothelial cells from umbilical veins or coronary arteries with vitamin C (1 – 100 µM, 24 h) led to an up to 3-fold increase of agonist-induced NO production measured as the formation of citrulline (co-product of NO) and cGMP (product of the NO-activated soluble guanylate cyclase). The effect was specific, saturated at 100 µM and followed a similar kinetics as seen for the uptake of vitamin C into the cells. Vitamin C did not induce the expression of the NO synthase (NOS) protein nor enhance the uptake of the NOS substrate L-arginine into the cells. The effect of vitamin C on NO formation was abolished when intracellular levels of the NOS cofactor tetrahydrobiopterin (BH4) were increased by coincubation of cells with sepiapterin. Accordingly, pretreatment of endothelial cells with vitamin C (1-100 µM, 24 h) revealed an up 3-fold increase of intracellular BH4 levels which was not due to an enhanced BH4 synthesis. Neither the mRNA expression nor the activity of the rate-limiting enzyme in BH4 biosynthesis, GTP cyclohydrolase I, were altered by vitamin C. The increase of BH4 levels in endothelial cells coincubated with cytokines and vitamin C was associated with a decrease of 7,8-dihydrobiopterin and biopterin in cells and cell supernatants indicating that vitamin C led to a chemical stabilization of BH4. These results suggest that saturated vitamin C levels in endothelial cells are necessary to protect BH4 from oxidation and to provide optimal conditions for cellular NO synthesis.

SESSION V
THE ANTIOXIDANT VITAMINS C AND E

The vitamin E atherosclerosis prevention study (VEAPS): Reduction in LDL oxidation without effects on atherosclerosis progression rates?

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Background. Epidemiological studies have demonstrated an inverse relationship between vitamin E intake and cardiovascular disease (CVD) risk. In contrast, randomized controlled trials have reported conflicting results as to whether vitamin E supplementation reduces atherosclerosis progression and CVD events.

Methods and Results. The study population consisted of men and women, 40 years of age or greater, with LDL-cholesterol $\geq$130 mg/dL, and no clinical signs or symptoms of CVD. Eligible participants were randomized to vitamin E (DL-α-tocopherol) supplementation at 400 IU per day or placebo and followed every three months for an average of three years. The primary trial end point was the rate of change in the common carotid artery far wall intima-media thickness (IMT) assessed by computer image processed B-mode ultrasonograms. A mixed effects model using all available determinations of IMT was used to test the hypothesis of treatment differences in IMT change rates. Compared to placebo, vitamin E supplementation significantly raised plasma vitamin E levels ($p<0.0001$), reduced circulating oxidized LDL ($p=0.03$), and reduced LDL oxidative susceptibility ($p<0.01$). However, vitamin E supplementation did not reduce the progression of IMT over a 3-year period as compared with subjects randomized to placebo.

Conclusions. The results are consistent with previous randomized controlled trials and extend the null results of vitamin E to the progression of IMT in healthy men and women at low risk for CVD.

Is there a role for vitamin E in the prevention of atherosclerosis?

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Atherosclerosis, the most common underlying cause of coronary heart disease (CHD), represents a state of heightened 'oxidative stress' characterized by aortic lipid and protein oxidation. Animal studies and observational human cohort studies have been interpreted as supporting a role for antioxidants, particularly vitamin E, in the prevention of CHD, and inhibition of LDL oxidation is commonly thought to be the underlying mechanism. Secondary prevention studies of relatively short duration of follow-up and employing small populations have shown some benefit from vitamin E. However, vitamin E does not reduce the incidence of coronary events in primary prevention studies, and fails to provide benefit in larger randomized trials. Recent studies with antioxidant combinations also show no benefit.

Basic research revealed that the molecular action of vitamin E is more complex than previously thought and that the vitamin alone does not necessarily protect lipoprotein lipid from oxidation. Indeed, in human arteries oxidized lipids accumulate and are likely formed in the presence of vitamin E that remains essentially intact as disease develops. Also, oxidized lipids characteristic for radical-mediated lipid peroxidation accumulate after non-oxidized lipids during atherogenesis, and the pattern of oxidation products of vitamin E suggests that radical (*ie,* 1*e*-) oxidants are less important than 2*e*-oxidants such as hypochlorite that can convert LDL into an atherogenic lipoprotein via pathways unaffected by vitamin E.

Thus, convincing proof of a positive effect of vitamin E supplements on CHD is presently not available. Mechanistic studies suggest that supplementing vitamin E alone may not be beneficial if lipoprotein lipid peroxidation and/or oxidative events involving 2*e*-oxidants are important causes of atherosclerosis.

Randomised trial of cholesterol-lowering therapy and of antioxidant vitamins in 20,536 people at increased risk of coronary heart disease death

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The Heart Protection Study assessed the effects of cholesterol-lowering therapy and of antioxidant vitamin supplementation in various patient categories for which there had been uncertainty about the value of such treatment. Patients aged 40-80 with a history of occlusive vascular disease or diabetes were eligible provided their own doctors did not consider statin therapy clearly indicated. Between July 1994 and May 1997, 20,536 patients were recruited in 69 UK hospitals. Previous MI was reported by 8510 (most of whom were elderly, female or had "low" cholesterol levels) and some other CHD by 4876. Among the 7150 with no history of CHD, 1820 reported a previous stroke or TIA, 2701 some other peripheral artery disease, and 3982 diabetes (with overlap between these categories). There were 5082 women and 15,454 men, with 4892 aged 65-69 and 5805 aged 70-80. Total cholesterol was <5.0 mmol/l (194 mg/dl) in 4072, and LDL cholesterol was <3.0 mmol/l (116 mg/dl) in 6793. Participants were randomly allocated simvastatin 40 mg daily or matching placebo for 5_ years. On average during the study, about one-sixth of participants allocated simvastatin stopped taking statin therapy and one-sixth of those allocated placebo started taking a statin, yielding an average LDL difference of 1.0 mmol/l (39 mg/dl). Using a factorial design, half of each treatment group was also randomly allocated antioxidant vitamins (600 mg E, 250 mg C, 20 mg beta-carotene daily) and half allocated placebo.

Preliminary analyses involve non-fatal MI or CHD death ("total CHD" events) in 2148 participants, non-fatal or fatal stroke in 1069, "major vascular events" (total CHD, total stroke or any

revascularisation) in 4648, cancer (excluding non-melanoma skin) in 1636, and 2831 deaths from all causes. No beneficial or adverse effects were seen with the vitamins. Statin therapy reduced total and vascular mortality. Allowing for non-compliance, simvastatin 40 mg daily reduced "major vascular events" by at least one-third in a wide range of high-risk patients (including women, people aged 70+, or with LDL <3.0 mmol/l, or with diabetes but no CHD).

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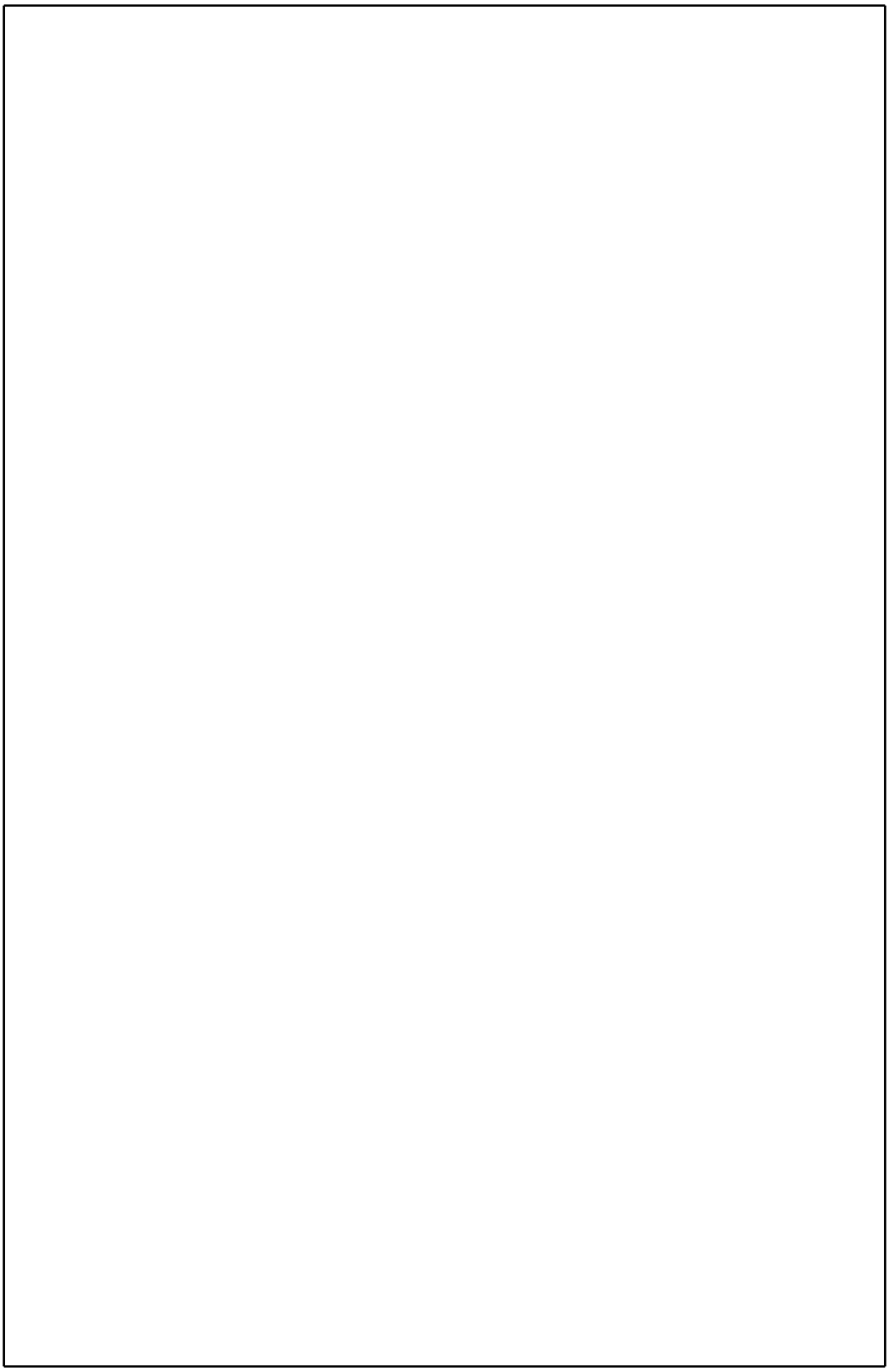
Preliminary results are described in greater detail on www.hpsinfo.org



SESSION VI
THE ANTIOXIDANT VITAMINS C AND E

Vitamin E (E) and enhancement of the immune response in the aged: Cellular and molecular mechanisms

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The incidence of neoplastic and infectious diseases is increased in the elderly, as is the resulting morbidity and mortality. The well-documented age-associated decline in T cell function is an important contributor to the increased incidence of and mortality from these diseases. We have previously shown that E supplementation of elderly humans will significantly improve T cell mediated function including their response to vaccination.(1) Furthermore, E supplementation of old mice significantly improved their resistance to influenza infection.(2) The E-induced enhancement of T cell function was mediated through an increase in production of the cytokine IL-2, leading to enhanced proliferation of T cells from aged animals and humans. This was further shown to be, in part, due to E-induced reduction in T-cell suppressive factor prostaglandin (PG) E2,(3) the production of which by macrophages (MØ) is increased with age.(4) Vitamin E reduced macrophage PGE2 production through a reduction in peroxynitrite formation.(5) In addition, a direct effect of E on T cell function, not mediated through its PGE2 lowering effect on MØ, has been suggested.(6) To evaluate the mechanism of a direct immuno-enhancing effect of vitamin E on T cells, we showed that the age-related defects in T cell function were only observed within naive (CD44lo) T cells.(7) Furthermore, E increased both cell dividing and IL-2 producing capacity of naive T cells from old mice, with no effect on memory (CD44hi) T cells. This effect of E was not due to a decrease in apoptosis nor to a change in calcium mobilization of naïve T cells in the aged mice. Thus, in addition to its lowering of MØ PGE2 production, E enhances T cell function directly by enhancing the cell dividing and IL-2 producing capacity of naïve cells. Further studies are needed to determine the signaling mechanisms involved in E-induced up-regulation of naïve T cells function in the aged.

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α-Tocopherol, oxidative stress, inflammation and diabetes

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The diabetic state confers an increased propensity to accelerated atherogenesis. In addition to the established risk factors of hypertension, dyslipidemia and a pro-coagulant state, there is evidence for increased oxidative stress and inflammation in diabetes. Increased oxidative stress is manifested by increased lipid peroxidation, increased F2-isoprostanes and increased DNA damage. Evidence for increased inflammation includes increased monocyte activity (cytokine release, IL-1, IL-6 and TNF-α), increased monocyte adhesion to endothelium and increased levels of plasma C-reactive protein, the prototypic marker of inflammation. Most importantly, alpha tocopherol therapy, especially at high doses, clearly show a benefit with regards to LDL oxidation, isoprostanes and a decrease in inflammatory markers such as C-reactive protein pro-inflammatory cytokines and PAI-1 levels. Thus, it appears that in diabetes α-tocopherol therapy could emerge as an additional therapeutic modality.

Potential adverse effects of vitamins C and E

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High dietary intakes of the antioxidant vitamin C have been associated with reduced risk of degenerative diseases. Very high amounts of vitamin C (2-4 g/day) are generally well tolerated biologically but individuals with renal or iron overload pathologies may react adversely to supplemental vitamin C. The lipophilic antioxidant vitamin E is also generally considered safe and without side effects at dosages less than 1000 mg/d. Since vitamin E decreases platelet adhesion as well as inhibits the oxidation of low-density lipoproteins, vitamin E has been promoted for the prevention and treatment of coronary artery disease. However, long-term ingestion of vitamin E (50 mg/d for over 5 y) has been associated with a slight risk of hemorrhagic stroke. Patients receiving warfarin therapy should be discouraged from using vitamin E supplements, and vitamin E should be discontinued in the perioperative period in patients requiring surgery. Investigations in smokers suggest that high doses of vitamin E may decrease plasma vitamin C in individuals with low intakes of vitamin C thereby altering the redox balance in vivo and causing cytotoxic effects. Also, a recent report demonstrated an adverse interaction between antioxidant supplementation (including 500 mg vitamin C and 364 mg vitamin E) and statin/niacin therapy in patients with coronary artery disease. Supplemental vitamin C and vitamin E provide physiologically important antioxidant effects in vivo, yet knowledge on potential adverse effects in some populations is important for determining the risk-to-benefit ratio of supplementation.

Serum ascorbic acid and disease prevalence: The third national health and nutrition examination survey (NHANES III)

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Ascorbic acid is an essential nutrient and important water-soluble antioxidant with a number of biological effects. We used data collected from the Third National Health and Nutrition Examination Survey (NHANES III), a large probability sample of Americans conducted between 1988-1994, to examine the relation of ascorbic acid status to disease risk factors and to health outcomes. We specifically examined the relation of serum ascorbic acid levels to cardiovascular disease (self-reported angina, myocardial infarction, or stroke); gallbladder disease (including asymptomatic gallstones detected by abdominal ultrasonography); blood lead levels and lead poisoning; bone density and self-reported fractures of the hip, wrist, and spine; seropositivity to *Helicobacter pylori,* a bacterial risk factor for peptic ulcer disease and possibly gastric cancer; and to a number of non-traditional cardiovascular disease risk factors, including serum levels of albumin, creatinine, homocysteine, uric acid; and plasma fibrinogen, and leukocyte count. The results of these analyses will be discussed, as well as, the limitations related to the use of data from NHANES III.

Vitamin C status and mortality in US adults

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Vitamin C may play a role in prevention of cardiovascular disease (CVD) and cancer, the two leading causes of mortality in the United States. We used data from a nationally representative sample of US men (n=3,347) and women (n=3,724) 30-74 years of age to test whether an association existed between serum ascorbate concentration and mortality, and if it was modified by cigarette smoking or gender. Serum ascorbate concentrations were measured in the Second National Health and Nutrition Examination Survey (NHANES II), 1976-80, and vital status of adult participants was ascertained after 12-16 years. Risk of death was estimated using Cox proportional hazards models and adjusted for age at baseline exam, race (African American, other), highest attained education level (<12, 12 years), number of cigarettes smoked at baseline, weekly frequency of consuming alcohol, diabetes (yes, no), serum total cholesterol, systolic blood pressure, and body mass index. Men in the lowest (<28.4_mol/L (0.5 mg/dl)) compared with the highest serum ascorbate quartile (73.8 _mol/L (1.3 mg/dl)) had a 57% increased risk of dying from any cause (RR=1.57, 95% C I=1.21-2.03) and a 62% increased risk of dying from cancer (R R=1.62, 95% CI=1.01-2.59). No significantly increased risk existed among men in the middle two quartiles for these outcomes, nor was there an increased risk of CVD mortality in any quartile after adjustment for established CVD risk factors. Among women, there was no association between serum ascorbate quartile and mortality.

These findings were consistent when analyses were limited to non-smokers or further to adults who never smoked, indicating that the observed relationships were not confounded by cigarette smoking. These data suggest that men with low serum ascorbate concentrations may have an increased risk of mortality, likely due to an increased risk of dying from cancer. In contrast, serum ascorbate concentrations were unrelated to mortality among women.

SESSION VII
NEW HORIZONS ON CAROTENOID RESEARCH

The role of carotenoids in age-related macular degeneration: Where are we now, and where do we go from here?

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In recent years, carotenoids have emerged as important protective factors against visual loss from age-related macular degeneration (AMD), the leading cause of irreversible blindness in the developed world. The xanthophyll carotenoids lutein and zeaxanthin are concentrated specifically in the macula of the human retina, and epidemiological studies have shown that diets high in these compounds appear to be protective against AMD, presumably through antioxidant and light screening mechanisms. More recently, the Age-Related Eye Disease Study (AREDS) has shown in a large prospective randomized placebo controlled trial that supplementation with high doses of a combination of β-carotene, zinc, vitamin E, and vitamin C can decrease the rate of progression to advanced AMD in high risk individuals by over 25%, consistent with the hypothesis that AMD is in part a disease resulting from excessive oxidative stress. The biochemical mechanisms responsible for the specific uptake and metabolism of the macular carotenoids in the human macula will be reviewed, and our recent results of in vivo clinical studies of macular carotenoid measurements in normal and AMD subjects by resonance Raman spectroscopy will be presented. Future directions to clarify the therapeutic roles of lutein, zeaxanthin, and β-carotene in AMD will be discussed.

Roles of antioxidants and DNA repair in regulating oxidative DNA damage

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We have examined the influence of antioxidants on the level of oxidative DNA damage in several human trials. If lymphocytes are treated with H_2O_2 *in vitro*, the extent of induction of DNA breaks is a measure of the antioxidant status of the lymphocytes. Alternatively, the effect of antioxidants on the level of endogenous DNA base oxidation can be assessed. How low is this background level? Until recently, 8-oxoguanine was estimated at anything from 1 to 1000 *per* 10^6 unoxidised bases. The variation depends on the technique used; GC-MS tends to give the highest estimates, HPLC-ECD rather less, but lowest of all are the values obtained by incubating DNA with repair endonucleases to convert oxidised bases to breaks which are then measured by alkaline elution, alkaline unwinding or the comet assay. ESCODD, the European Standards Committee on Oxidative DNA Damage, has made a concerted attempt to resolve the methodological problems and reach consensus on the true background level. It is now clear that the higher estimates resulted from oxidation of guanine during preparation of the DNA for analysis. Antioxidants and DNA repair both help to maintain the low steady-state level of damage. Little is known about the modulation of repair activity by dietary factors. We have begun to look at the possibility that enhancement of repair by antioxidants or other phytochemicals might account for the protection afforded by fruits and vegetables against cancer. Lymphocytes isolated after a week of β-carotene supplementation appear to repair oxidative damage faster than do lymphocytes isolated before the supplementation. A more reliable test is the assessment of repair capacity in lymphocyte extracts in an *in vitro* assay. This has shown convincing stimulation of repair by supplementation with antioxidant-rich foods.

β-Carotene and lycopene in oral sun protection

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When skin is exposed to UV-light, erythema is observed as an initial reaction. Photooxidative processes play a role in the pathobiochemistry of erythema formation, and protective effects of carotenoids against sunburn have been demonstrated (1). When β-carotene was applied as such or in combination with α-tocopherol for 12 weeks, erythema formation induced with a solar light simulator was significantly diminished from week 8 on.

Such protective effects are also achieved with a diet rich in lycopene (2). Ingestion of tomato paste, corresponding to a dose of 16 mg lycopene/day over 10 weeks, led to increases in serum levels of lycopene and total carotenoids in skin. At week 10, erythema formation was significantly lower in the group that took the tomato paste as compared to the control group. No significant difference was found at week 4 of treatment. Thus, protection against UV-light-induced erythema can be achieved by ingestion of a commonly consumed dietary source of lycopene. Dietary antioxidants may be used to increase the basal protection via systemic distribution.

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SPECIAL LECTURE

Historical critical evaluation of free radical and antioxidant research

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It has been almost 50 years since Rebecca Gerschman and colleagues proposed that O_2 poisoning and x-irradiation had a common mechanism, i.e., free radical formation. Just 2 years later, in 1956, Denham Harman proposed that free radicals could damage functionality in humans, resulting in inappropriate aging or the development of chronic diseases. While these investigators and others were elucidating free radical effects, others workers from the food technology area such as Al Tappel were concentrating on the initiation, propagation and termination of lipid oxidation. By the late 1940's and early 1950's, it was clear that the lipid oxidation observed in vitamin E-deficient animals, could be inhibited by a series of phenolic compounds including vitamin E. The next step with respect to nutritional antioxidants was the observation of the reduction of oxidized vitamin E by vitamin C, which in turn could be recycled by biological reductants such as NADPH or GSH.

While these developments clarified the roles of "antioxidant vitamins", the discovery of $O_2{\cdot}^-$ by Fridovich and McCord in 1968 and the subsequent characterization of superoxide dismutase opened up the field of "antioxidant enzymes". However, we are now at the state where we have begun to realize that the functional term "antioxidant' need not explain all of the biological activities of this large class of nutrients and enzymes.

SESSION VIII

REDOX REGULATION OF SIGNAL TRANSDUCTION

Thioredoxin targets involved in subcellular traffic

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Human thioredoxin (TRX) was originally identified as Adult T-cell leukemia-derived factor (ADF) produced by human T-cell leukemia virus type I (HTLV-I) positive T-cell lines. TRX/ADF interacts with multiple target molecules in mammalian cells, including transcription factors (AP-1, NF-κB) as well as signaling molecules (ASK-1, Ref-1). We recently identified thioredoxin-binding protein-2/vitamin D3 up-regulated protein 1 (TBP-2/VDUP1) as a negative regulator of TRX. In many of the T cell lines virally transformed by HTLV-I, there was a reciprocal expression between TRX and TBP-2. TBP-2 mRNA was suppressed in four HTLV-I positive T-cell lines (ATL-2, Hut102, MT-1 and MT-2), but not in HTLV-I negative T-cell lines (Jurkat and Molt4). Forced expression of TBP-2 by TBP2 cDNA or introduction of TAT-TBP2 protein exhibited a marked reduction in cell proliferation rate, suggesting that TBP-2 protein acts as a tumor suppressor. TRX dominantly localizes in cytosol, and readily translocates to the nucleus in response to oxidative stress. As TBP-2 is localized in nuclear compartment, we hypothesized that TBP-2 and TRX may be translocated either independently or as a complex to regulate nuclear events. We also identified a candidate molecule that interacts with TBP-2 in relation to its nuclear localization. We will discuss the functional significance of the interaction between TRX and its target molecules depending on the differentiation stage and lineage of the cells in association with the regulation of cell growth and cell cycle.

Diversity of redox activation pathway for NFκB in response to environmental injuries

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The cellular redox environment has been increasingly recognized as a critical component of stress-induced cellular responses and disease. Inherent in these responses are reactive oxygen species which inflict direct cellular damage in addition to acting as intracellular second messengers modulating signal transduction pathways. In the present study we have begun to elucidate two redox-sensitive pathways capable of activating NFκB by either serine or tyrosine phosphorylation of IkB-α. In vitro and vivo models of ischemia/reperfusion have demonstrated that tyrosine, but not serine, phosphorylation of IkB-alpha occurs prior to induction of NFκB and that this pathway is sensitive to the level of intracellular superoxides. In contrast, pro-inflammatory stimuli such as TNF-alpha or LPS challenge activates NFκB through IKK mediated serine phosphorylation of IkB-α. The specific redox interactions required for activation of NFκB following pro-inflammatory stimuli appears to be at least in part facilitated by hydrogen peroxide mediated activation of the IKK-α subunit in the IKK complex. To begin to better understand the mechanisms that control NFκB activation and determine cell fates following cellular injury, we utilized a number of recombinant adenoviral vectors encoding antisense mRNA or serine and tyrosine phosphorylation mutant cDNAs of IkB-α. NFκB activation in these studies was monitored using an NFκB-responsive luciferase reporter also encoded within a recombinant adenoviral vector. Interestingly, activation of NFκB following I/R or pervanadate treatment occurred through a specific pathway mediating IkB-α phosphorylation on tyrosine 42. In this context, activation of NFκB played an anti-apoptotic role and

increased cell survival. In contrast, activation of NFκB following TNF-α or UV exposure was pro-apoptotic and controlled by serine 32/36 phosphorylation of IkB-α. Taken together, these findings underscore the complexities associated with redox activation of NFκB and the consequences of that activation.

Sptrx-1, a novel thioredoxin expressed during mammalian sperm tail elongation

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Thioredoxins are a growing family of proteins that function as general protein disulfide reductases. Thioredoxins are maintained in their reduced active form by the flavoenzyme thioredoxin reductase at the expense of NADPH. Sptrx-1 is the first member of the thioredoxin family with a tissue specific distribution and is exclusively found in mammalian spermatozoa. Sptrx-1 is composed of a N-terminal domain organized as repetitions of a 15 amino acid residue motif and a C-terminal domain typical of thioredoxins. Northern blot and *in situ* hybridization analyses identify Sptrx-1 mRNA in the round and elongating spermatids of testis seminiferous tubules. Recombinant human Sptrx-1 displays both thioredoxin reductase-dependent reducing activity and oxidizing activity in the presence of selenite, *in vitro*. By mass spectrometry and gel filtration chromatography, we have shown that Sptrx-1 has an oligomeric structure, not maintained by disulfide bonds. Western blot analysis identifies the protein only in mammalian testis and spermatozoa extracts. By light microscopy, we find Sptrx-1 in the developing tail of the elongating spermatids and immunogold electron microscopy identifies the protein associated to the two longitudinal columns of the sperm tail fibrous sheath. However, this tail localization is transient and Sptrx-1 disappears from the sperm tail after its formation. Based on Sptrx–1 expression pattern during spermiogenesis and its dual reducing/oxidizing activity, we propose Sptrx-1 as a key regulator for the correct formation of the fibrous sheath, an essential structure required for sperm flagellar movement. The role of Sptrx–1 in the male infertility phenotype will be discussed.

Cystine/glultamate exchange transporter: occurrence and redox regulation of its expression

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We have described in cultured mammalian cells a Na^+-independent anionic amino acid transport system highly specific for cystine and glutamate. This system is designated as system x_c^- and is composed of two subunits, xCT and 4F2hc. System x_c^- is an exchange agency and the anionic form of cystine is transported in exchange for glutamate. Cystine taken up by the cells is reduced to cysteine, a rate-limiting precursor for glutathione (GSH) synthesis. Thus, GSH level is regulated by system x_c^- activity in cultured cells and as a consequence of the transport of cystine, system x_c^- affects the extracellular balance between cystine and cysteine. The activity of system x_c^- is induced by electrophilic agents. This induction involves transcriptional upregulation of subnit xCT. An electrophile response element (EpRE) has been found in 5' flanking region of xCT gene and this element and the transcription factor Nrf2 were required for the induction. System x_c^- activity is also induced upon cystine starvation. In this case cells are exposed to severe oxidative stress due to GSH depletion. A novel element neighboring EpRE was involved in this transcriptional upregulation of xCT. Induction of system xc- is thought to be an adaptive response of the cells to the oxidative stress. Northern blot analysis showed that xCT message was expressed relatively strongly in brain. *In situ* hybridization analysis revealed the strong expression of xCT message in meninges and some circumventricular organs, suggesting an important role of system x_c^- in redox balance in cerebrospinal fluid.

Session IX
Oxygen, Antioxidants, and Redox Signaling

Superoxide dismutases and cancer: Evidence for two distinct types of lung cancer

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RNA was isolated from tumor and surrounding healthy tissue from ten patients with pulmonary adenocarcinoma, and subjected to analysis by Affymetrix GeneChip (Human Genome U95Av2) Array, revealing the expression levels of more than 12,000 known genes. When expression levels of the cytosolic *SOD1* were correlated with those of the mitochondrial *SOD2*, a distinctive relationship was revealed. Five of the subjects overexpressed the *SOD1* gene by 54% ($p < 3 \times 10\text{-}5$) and underexpressed the *SOD2* gene by 65% ($p < 0.0003$) (which we designate Type I), while the other five expressed both *SOD1* and *SOD2* at normal levels (Type II). These two distinct populations differed significantly in expression levels of a large number of other genes associated with malignancy. E.g., Type I cancers significantly overexpressed glutathione-S-transferase, thioredoxin, G6PD and interferon regulatory factor 4, while Type II cancers expressed normal amounts of these genes. Conversely, Type II cancers over-expressed such genes as mucin, vascular endothelial growth factor (VEGF), urokinase plasminogen activator receptor (uPAR), and matrix metalloproteinase 2, all associated with metastasis and angiogenesis, while Type I cancers were normal for these genes. The data suggest that about half of lung adenocarcinomas are in a state of oxidative stress. The ones with normal SOD expression, however, would appear to be more prone to metastasis.

Translational regulation of MnSOD by an RNA-binding protein: Role of tyrosine phosphorylation

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There are several animal models of oxidant stress in which MnSOD is regulated, at least in part, at a post-transcriptional level. After demonstrating the presence of a specific protein that binds to the 3'UTR of MnSOD mRNA, we delimited a 41-base cis element involved in binding the protein; and we determined that the 3'UTR response element functions as a translational enhancer through its interaction with the MnSOD RNA-binding protein by facilitating the formation of the translation initiation complex. More RNA-binding activity, MnSOD protein synthesis, and enzyme activity are present in the lungs of neonatal rats that are tolerant to hyperoxia compared with lungs of non-tolerant adult rats. Taken together, these findings support the hypothesis that the ability to increase MnSOD expression in response to oxidant stress is modulated by the action of the RNA-binding protein that increases translational efficiency (protein synthesized per RNA). Furthermore, the cis element is capable of regulating the translation of a heterologous RNA, suggesting it may be useful in designing vectors for expression of recombinant proteins in vitro or in gene therapy. Cell culture and in vitro studies indicate that MnSOD RNA-binding protein activity is regulated by a tyrosine phosphorylation switch mechanism and that the dephosphorylated protein is more active in binding the MnSOD 3'UTR response element resulting in an increase of MnSOD protein synthesis without an increase in MnSOD RNA. We now propose a model in which signal transduction by reactive oxygen species alters the activity of protein tyrosine phosphatase/kinase pathways that subsequently modulate MnSOD expression by controlling MnSOD RNA-binding protein activity.

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Revisiting the neuroprotective properties of vitamin E

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Glutamate-induced oxidative stress and toxicity represents a major event in stroke and several other neurodegenerative disorders. Previously we have reported that nM α-tocotrienol (T3), but not α-tocopherol or any other antioxidant tested, blocked glutamate induced death by suppressing glutamate-induced early activation of c-Src kinase in HT4 cells (*JBC* 275:13049-55, 2000). The objective of the present study was two-fold: first, to test whether the neuroprotective effect of nM T3 applies to primary neurons; secondly, we sought to further characterize the molecular targets of T3 in the death path. Consistent with observations from HT4 cell line, nM (25-100) T3 protected primary fetal cortical neurons from glutamate- as well as BSO-induced death. In pregnant rats supplemented with a T3 rich fraction of palm oil, T3 levels in the mother and fetal brain increased by 5- and 20-fold, respectively. High-density microarray studies were performed to identify vitamin E sensitive genes in the developing brain. Analysis of the signal transduction pathways revealed that 12-lipoxygenase activity is induced in neuronal cells in response to glutamate treatment and that this response is a key player in the execution of death. Glutamate treatment resulted in 12-HETE formation and authentic 12-LOX inhibitors prevented glutamate-induced cell death. In both cell culture as well as purified enzyme studies nM T3 exhibited potent 12-LOX inhibitory activity. Primary neurons isolated from 12-LOX knockout mice were resistant to glutamate-induced death confirming a key role of 12-LOX in the execution of death. Results from our laboratory identify tocotrienol as a highly potent neuroprotective form of vitamin E that is indeed bio-available to the brain when fed orally.

Multiple and tissue specific actions of tocopherol-transfer-protein gene *in vivo*

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Mutations in the gene for tocopherol-transfer-protein (TTP) in humans result in neurodegenerative diseases. The patients have decreased amounts of vitamin E in plasma and in tissues and show the importance of the TTP gene in vitamin E homeostasis. Normalization of plasma and tissue vitamin E through large increases in dietary vitamin E only partially reverses the pathologies of TTP defect suggesting that the TTP gene may have additional functions *in vivo*. We have utilized transgenic, TTP-null mice and high-density oligonucleotide arrays to obtain comprehensive and quantitative role of the TTP gene *in vivo*. Gene expression profiles of ~5000 distinct genes from liver and brain cortex from TTP-null and wild type mice were determined and compared. Gene expression data revealed tissue specific effects of TTP gene. Liver, the primary organ of vitamin E uptake and distribution showed a net decrease in the expression 11 genes; these included the genes encoding rate controlling enzymes in the synthesis of long chain fatty acids and steroid metabolism. The activities of several genes were induced and included those encoding enzymes for the synthesis of sphingolipids and glutathione metabolism. In brain cortex, a net increase in the expression of genes was detected in the TTP-null mice compared to the wild type controls. A large number of induced transcripts encoded the enzymes of the mitochondrial electron transport chain and its regulatory proteins. A number of transcripts were down regulated in cortex of TTP-null mice; these included two mRNAs for protein kinase C. Particularly notewor-

thy was the repression of mRNA for proteolipid protein that is essential for the synthesis of myelin. The latter observation predicts an important role of TTP-gene for normal functioning of myelinated neurons. Collectively, gene expression profiling of TTP–null mice has identified specific effects of the TTP-gene on the mouse transcriptome in two functionally distinct organs. The data identify probable genomic targets of vitamin E in vivo and suggest an essential role of this lipophilic antioxidant in cellular signal transduction, mitochondrial functions, and lipid and steroid metabolism.

SESSION XI

NEURODEGENERATION

Neuroprotective effects of natural extracts and NOS inhibitors against β-amyloid toxicity

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Accumulation of beta-amyloid (Aβ) peptides is one of the leading hypothesis to explain neurodegenerative processes that occur in Alzheimer's disease. Although mechanisms have yet to be fully established, it has been proposed that Aβ-mediated toxicity is associated with increases in reactive oxygen species levels.

Aβ and the NO donor peroxynitrite were able to increase nitric oxide (NO) production and decrease cellular viability in rat cortical cell cultures and these events were attenuated by L-NIL and 1400W - two type II NOS inhibitors – as well as by the NO scavenger carboxy-PTIO.

Similarly, the ginkgo biloba extract EGb 761 displayed neuroprotective abilities against Aβ- and NO-induced toxicity in rat hippocampal cell cultures, and these effects are likely attributable to antioxidant properties of its flavonoid constituents. The protective effects of EGb 761 were shared by polyphenols that are present in high amount in green tea and red wine.

Taken together, the data support the hypothesis that free radical production may account for Aβ neurotoxicity and suggest that antioxidant compounds may have therapeutic importance in neurological disorders where oxidative stress is likely involved.

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Signaling Oxidative Stress in Alzheimer's Disease

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The temporal association between oxidative stress and the hallmark pathologies of Alzheimer's disease (AD) demonstrates that oxidative stress is among the earliest events in the disease. Nonetheless, neither the consequence of oxidative stress nor how oxidative stress relates to other pathological features of the disease are clear at this point. To begin to address these issues, we investigated the p38 stress-activated protein kinase pathway in the pathogenesis of AD. In affected brain regions of individuals with AD, p38 kinase is abnormally activated and associated with neurofibrillary pathologies. By marked contrast, these brain regions exhibit a low level of diffuse p38 kinase in age-matched controls. The distribution and activation pattern of the upstream activator of p38, namely MKK6, is also altered in AD compared to control brain, suggesting that the entire p38 pathway is activated.

Given the complete overlap between p38 kinase and tau-positive neurofibrillary pathology *in situ*, we suspect that p38 may play a key role in disease pathogenesis. To explore this further, co-immunoprecipitation and dot blot assays indicated that the p38 kinase and tau are physically associated and thereby p38 is a likely candidate kinase for the phosphorylation of tau *in vivo*. Finally, since amyloid-β is thought to be the causative factor for the pathogenesis of AD and amyloid-β can act as an oxidative stressor, we suspected that amyloid-β may be responsible for the activation of p38 pathway. To investigate this possibility, using human M17 neuroblastoma and rat cortical primary neurons, we found that fibrillized amyloid-β induced the activation of p38 in a dose- and time-dependent manner, and, most importantly, that the activation of p38 mediated amyloid-β-induced cell death.

Taken together, these data indicate that oxidative stress-induced downstream events, mediated via p38 kinase, could play a key role in disease pathogenesis.

Flavonoids and protection against neurodegeneration

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Nutritional interventions, in this case, increasing dietary intake of fruits and vegetable, can retard and even reverse age-related declines in brain function and in cognitive and motor performance in rats. Our lab has shown that as Fischer 344 rats age their brains are increasingly vulnerable to oxidative stress. Dietary supplementation with fruit or vegetable extracts high in antioxidants (e.g., blueberry, BB, spinach, respectively) can decrease this vulnerability to oxidative stress as assessed *in vivo* by examining (reductions in neuronal signaling and behavioral deficits) and *in vitro* via H_2O_2-induced decrements in striatal synaptosomal calcium buffering. Examinations have also revealed that BB-supplementations (S) are very effective in antagonizing the age-related changes in neuronal signaling and behavior both by anti-inflammatory and antioxidant activities. In addition, there also appear to be direct effects on signaling. we have recently showed that BBS in mice transgenic for APP and PS-1 mutations reduced deficits in Y-maze performance and that the animals showing the fewest deficits in this behavior also showed up-regulated hippocampal PKC and ERK activity.

Mechanisms of oxidative stress-induced neuronal death: Potential targets for the neuroprotective effects of epicatechin

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Flavonoids have been reported to attenuate oxidative stress-associated cognitive decline and neuronal dysfunction implicated in neuronal loss in neurodegenerative diseases. OxLDL was used as a model for oxidative stress-induced neurotoxicity in cultured primary striatal neurones, leading to apoptosis as hallmarked by annexin-V-binding and DNA-fragmentation. The mechanisms of neurotoxicity involved a shift of the intracellular redox status towards oxidation and the activation of MAPK signalling cascades, in particular ERK1/2 and JNK. OxLDL caused a time- and calcium-dependent increase in levels of active ERK1/2 and JNK in neurones. OxLDL mediated the phosphorylation of c-Jun, the cleavage of procaspase-3 and an increase in caspase-3-like protease activity. These effects were inhibited by pre-exposure to low concentrations of epicatechin or kaempferol. The neuroprotective effects are seemingly independent of their H-donating capacity because epicatechin did not alter the oxidative shift of the cellular redox status mediated by oxLDL. Furthermore, a major *in vivo* metabolite of epicatechin, 3-*O*-methyl-epicatechin, a compound with an hydrogen-donating capacity substantially lower than that of epicatechin, showed a similar extent of protection against oxLDL-induced neurotoxicity. Additional data will be presented regarding the biological effects of major *in vivo* conjugates of epicatechin, including epicatechin-5-*O*-

β-D-glucuronide, with respect to: a) antioxidant properties; b) protection against oxidative stress-induced neuro-degeneration *in vitro*; c) identification of epicatechin/metabolites in the brain following supplementation.

Neurosteroid synthesis in neurodegeneration: An oxidative stress-mediated process

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Neurosteroids in rodents can originate from peripheral tissues or be locally synthesized in the brain. Human brain cells also have the ability to synthesize steroids from cholesterol. Oligodendrocytes are the source of pregnenolone in human brain. Astrocytes do not synthesize pregnenolone, nor do human neurons. There is potential for all brain cells to metabolize pregnenolone to other neurosteroids, including DHEA. Oligodendrocytes and astrocytes, but not neurons, make DHEA via an alternative pathway induced by treatment with Fe^{2+}, which acts on a yet unidentified precursor. In search of the regulation of DHEA formation, we observed that treating oligodendrocytes with β-amyloid (Aβ) peptide increases reactive oxygen species and DHEA formation. These effects of Aβ were blocked by vitamin E. To determine if this pathway exists in human brain, levels of DHEA in Alzheimer's disease (AD) patients and age-matched control brain tissues were measured and correlated with Aβ levels. DHEA was significantly higher in AD brain than control, and was highest in AD hippocampi. DHEA levels in AD serum were lower but not significantly different from controls. Treatment of control hippocampus and hypothalamus with FeSO4 increased DHEA synthesis, suggesting that levels of precursor are higher in control that in AD brain. This suggests that (i) an alternative precursor of DHEA is present in control brain and (ii) increased AD brain DHEA synthesis is triggered by increased levels of Aβ, iron and oxidative stress in the AD brain. Although DHEA

has been shown to protect neurons, locally made brain DHEA may act to protect glia, including reactive astrocytes that potentiate plaque formation in the AD brain, leading to accelerated AD pathology.

Session XII

Neurobiology and Nitric Oxide

A proteomics approach to study tyrosine nitration of mitochondrial complex I during cardiac ischemia-reperfusion

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Decreased mitochondrial complex I activity is widely reported following cardiac ischemia-reperfusion (I-R) injury, and may contribute to loss of contractile function. The reactive nitrogen species (RNS) nitric oxide (NO·) and peroxynitrite (ONOO-) are known to inhibit complex I *in-vitro*, and elevated RNS levels are reported in I-R. However, the mechanisms of complex I inhibition and the roles of RNS in I-R are unknown. It was thus hypothesized that RNS-mediated tyrosine nitration within complex I may be an underlying mechanism of mitochondrial dysfunction in I-R. We applied a novel proteomics approach using 2D blue-native electrophoresis to examine nitration of all mitochondrial complexes. In isolated heart mitochondria exposed to ONOO- (200µM) but not to NO· (2mM GSNO for 1hr.), dose-dependent nitration of 4 complex I subunits was observed. These subunits were identified by MALDI-TOF mass spectrometry. The degree of nitration correlated with loss of overall complex I enzymatic activity. Nitration of one subunit was also observed in mitochondria isolated from a Langendorff perfused rat heart model of I-R injury. In addition, mitochondria isolated from infarct zones of explanted human hearts showed complex I nitration. These data suggest that nitration of specific subunits of mitochondrial complex I is an important mechanism of mitochondrial dysfunction, and may be a mediator in the pathogenesis of cardiac I-R injury.

Oxidative apoptosis and novel targets for neuroprotection

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Oxidative stress is a downstream signal for the induction of nerve cell death. Apoptotic as well as necrotic processes can be directly or indirectly induced by free oxygen and nitrogen radicals. Indeed, oxidations are acknowledged as major player in the pathogenesis and progression of neurodegenerative disorders including chronic neurodegeneration (*e.g.* Alzheimer´s Disease; AD) and acute traumatic brain injury (*e.g.* cerebral Ischemia/Stroke). Lipophilic antioxidants prevent nerve cell death as induced by disease-associated neurotoxic stimuli or other apoptosis-inducing conditions. Besides vitamin E and other well-known lipophilic free radical scavengers, various endogenous hormones can also prevent oxidative stress. For instance, estrogen (17β-estradiol) has been found to be a powerful neuroprotectant via its antioxidant non-genomic activity as well as via its genomic effects. Estrogen as phenolic compound may serve as a "blue print" structure for the development of estrogen-related potent antioxidants lacking hormonal side effects. In addition, estrogen induces various intracellular signalling pathways and the transcription of estrogen target genes with neuroprotective functions including neurotrophic factors and anti-apoptotic proteins. In various experimental models of AD and stroke, estrogen and related compounds are highly neuroprotective.

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Neurotoxicity of NMDA: Novel therapeutic approaches *via* nitric oxide-mediated modulation

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S-nitrosylation (transfer of the NO group to protein thiol) leads to RS-NO formation and in some cases formation of disulfide bonds between neighboring cysteines (RS-SR). Over the past decade, beginning with an initial report on the *N*-methyl-D-aspartate type of glutamate receptor (NMDAR) (Lipton et al., Nature 1993), evidence has accumulated that S-nitrosylation can regulate the biological activity of a great variety of proteins. Increasing evidence, both physiological and chemical, suggests that the activity of the NMDAR can be inhibited by S-nitrosylation. Left uncurbed, overstimulation of the NMDAR leads to excessive Ca^{2+} influx, free radical generation and neuronal cell death by apoptosis if the insult is mild and by necrosis if the insult is more intense (Bonfoco et al., PNAS 1995; Budd et al., PNAS 2000).

Conventional NMDARs are probably tetramers composed of NR1 subunits and at least one NR2A-D subunit (although recent evidence has shown that other types of NMDARs also exist – see Chatterton et al., Nature 2002). From experiments on recombinant and native NMDAR subunits, we have learned that at least five cysteine residues can undergo S-nitrosylation resulting in a decrease in NMDAR activity: NR1 C744, C798; NR2A C87, C320, and C399 (Choi et al., Nature Neurosci. 2000). Of these, a single critical thiol (NR2A C399) exerts the predominant effect. Importantly, the other four residues are the same cysteines that influence redox signaling and high-affinity Zn^{2+} inhibition of the NMDAR (Choi et al., Nature Neurosci. 2000, J. Neurosci. 2001; Kim et al., Neuron 1999). S-Nitrosylation of these 4 cysteines appears to favor disulfide bond formation and enhances Zn2+ inhibition allosterically. S-

nitrosylation of Cys 399 also enhances Zn^{2+} inhibition of the receptor but in a mechanistically distinct manner from the other four cysteines involving enhanced Zn^{2+} binding and enhanced glutamate binding to its ligand recognition site on the receptor. The result of this increase in glutamate affinity is desensitization of the NMDAR, closure of NMDAR-associated channels, and consequent neuroprotection.

Comparative modeling of the NMDAR structure based on the crystal structures of homologous bacterial periplasmic binding proteins suggests that Cys 399 is located on a linker region separating the ligand-binding domain and the Zn^{2+}-binding regulatory domain of the NR2A subunit. The atomic model suggests the structural consequences of S-nitrosylation, and we propose a model to explain the observed inhibitory physiological effect of S-nitrosylation on NMDAR activity.

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Nitric oxide, mitochondria, and neurobiology

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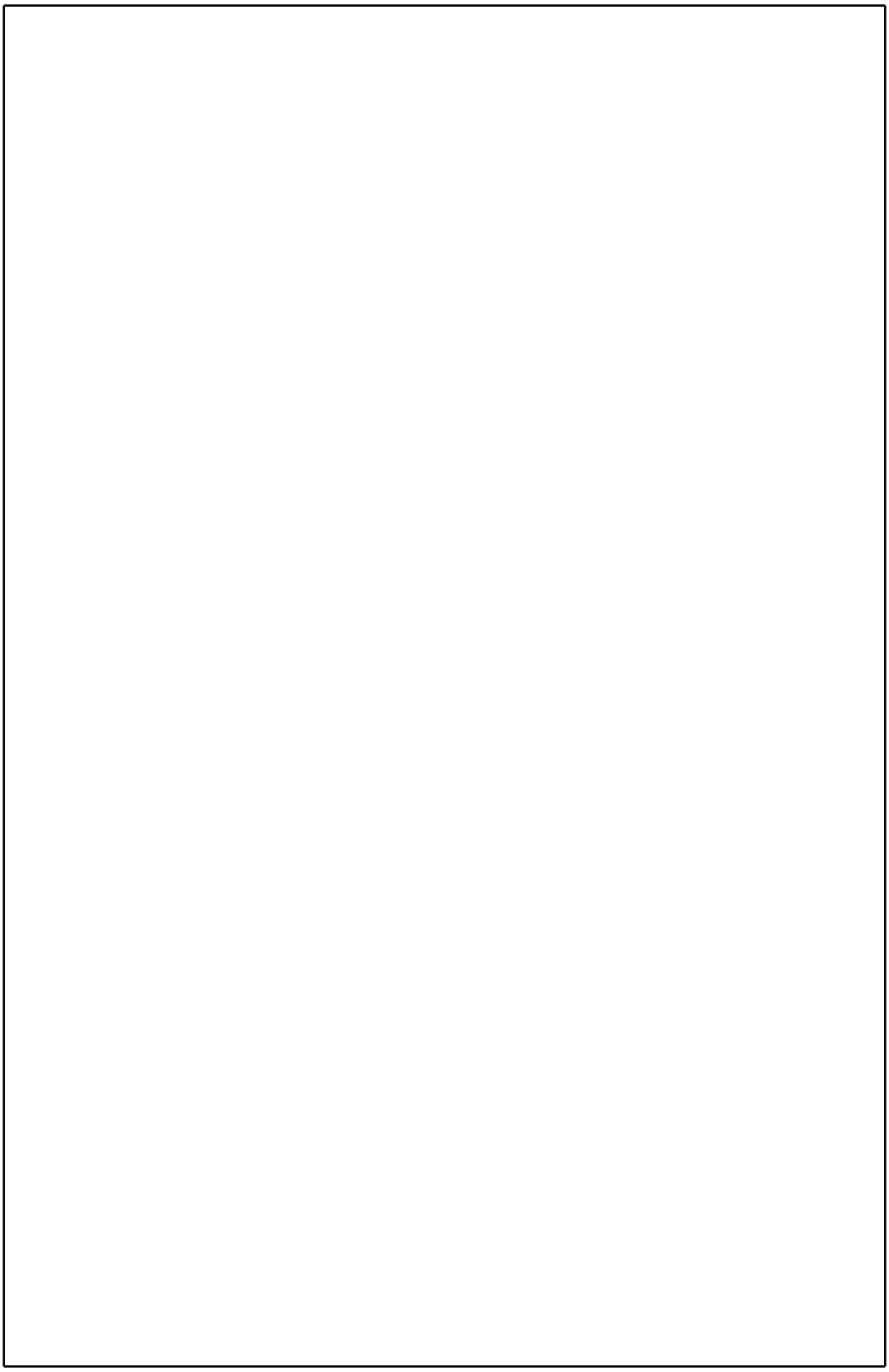
In the last years, different authors extensively studied the role of nitric oxide (NO) in the physiology and pathology of the nervous system. In brain, NO derives mainly from the activity of 160 kDa neuronal nitric oxide synthase (nNOS) although other isoforms, like constitutive eNOS and, in certain circumstances, iNOS may be present in neurons or glial cells. In addition, different nNOS splice isoforms retaining activity like 144 kDa nNOS and 135 kDa nNOSb or without activity, like 125 nNOSg, have been described. Moreover, different authors proposed the existence of mitochondrial NOS variants, specifically localized in mitochondria. In this way, we found a 144 kDa mtNOS in the purified rat brain mitochondria. The enzyme was localized by immune electron microscopy in the inner mitochondrial membrane, it has an specific activity of about 25% of the classic cytosolic isoform and is recognized by antibodies directed against the C-terminal domain (1095-1289) but not by antibodies against the N-terminal domain (0-188). The diversity of nNOS and of nNOS mRNA in the different tissues and developmental studies has been considered as “a major characteristic of nNOS gene expression”. In this way, we studied the developmental course of mtNOS in rat brain maturation. The mtNOS expression and activity increased from embryonic E15 to postnatal P10 and afterwards decreased to reach adult values, inversely to the course followed by classic nNOS; in addition, Mn-SOD activity paralleled the mtNOS modulation. In accord, a marked increase in the L-Arg-dependent mitochondrial production of hydrogen peroxide (H_2O_2) was observed at maximal mtNOS expression. The data propose specific functions of brain nNOS variants. While classic cytosolic nNOS has a prominent role in the

regulation of synapsis and in fired plasticity in the developed neural network, mtNOS should be rather a developmental enzyme, to modulate the redox transition from the embryonic proliferation phase to the structurally organized network, after synapsis connection and elimination. These findings could take part in neurodegeneration. Either increase or decrease in nNOS has been respectively reported in Parkinson's and Alzheimer in brain and other cell types; moreover, overexpressed neuronal mtNOS has been reported to be associated to non-neural pathology as heart damage in *mdx* dystrophic mice. It is suggested that nNOS variants have different functions in brain and other tissues and that, mitochondrial variants regulate O_2 uptake and redox status signaling for either proliferation, cell cycle arrest or apoptosis during maturation or, in disease.



KEYNOTE LECTURE

Delaying the mitochondrial decay of aging

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Mitochondria decay with age due to oxidation of RNA/DNA, proteins, and lipids. Oxidative mitochondrial decay is a major contributor to aging (1-5). We are making progress in reversing some of this decay in old rats by feeding them normal mitochondrial metabolites (acetyl carnitine and lipoic acid) at high levels. The principle behind this effect appears to be that with age increased oxidative damage to protein causes a deformation of structure of key enzymes, with a consequent lessening of affinity (K_M) for the enzyme substrate (6). The effect of age on the enzyme binding affinity can be mimicked by reacting it with malondialdehyde (a lipid peroxidation product). Feeding the substrate (acetyl carnitine) with lipoic acid, a mitochondrial antioxidant, restores the velocity of the reaction, Km for acyl carnitine transferase, and mitochondrial function (6). In old rats (*versus* young rats) mitochondrial membrane potential, cardiolipin level, respiratory control ratio, and cellular O_2 uptake are lower; oxidants/02, neuron RNA oxidation, and mutagenic aldehydes from lipid peroxidation are higher (3, 6-8). Ambulatory activity and cognition declines with age (7, 8). Feeding old rats acetyl carnitine and lipoic acid for a few weeks restores mitochondrial function; lowers oxidants, neuron RNA oxidation, and mutagenic aldehydes; and increases rat ambulatory activity and cognition (as assayed with the Skinner box and Morris water maze) (6-13).

Common micronutrient deficiencies accelerate mitochondrial decay. Heme biosynthesis is predominantly in the mitochondria. Interfering with heme synthesis causes specific loss of Complex IV with consequent release of oxidants (14, 15). Iron deficiency (20% of menstruating women) also causes release of oxidants and mito-

chondrial decay (16) presumably through lack of heme (14). Vitamin B6 deficiency (10% of Americans) would also cause a heme deficiency (14). It is inexcusable that anyone in the world is deficient for a vitamin or mineral, at great cost to health, when insurance, a daily multivitamin/mineral pill, costs less than $10/yr. The requirements of the old for vitamins/metabolites will differ from that of the young.

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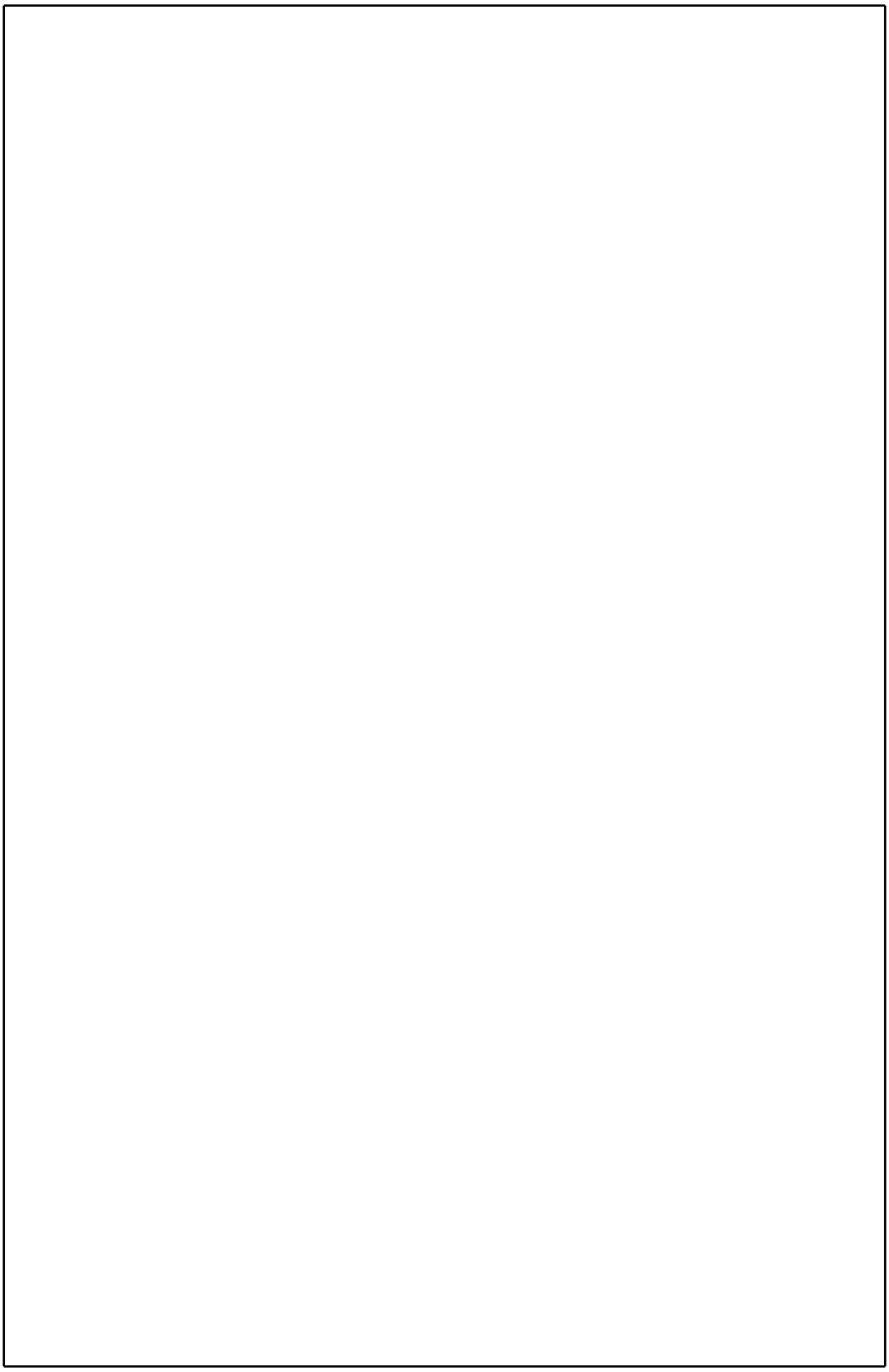

POSTERS

Bioprospection of protective polyphenolic compounds from acetone fraction of *Terminalia bellerica* using Ames assay

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Nature has provided a complete storehouse of remedies to cure all ailments of mankind. . As a result of man's inquisitive nature to that, today we possess many effective means of ensuring health care. The botanical derivatives, such as alkaloids, glycosides, oils, gums and tannins stand high in priority for preventing major diseases, including cancer, tuberculosis and Alzheimer's disease. Recent research has highlighted the immense importance of secondary metabolites because of their antmutagenic/anticarcinogenic properties. *Terminalia bellerica* is an important medicinal tree, the fruit of which contains a large amount of polyphenols and tannins. It is useful in treating eye diseases, adipose disorders, pain in passage of urine as well as overabundance of phlegm and blood. The acetone extract of *T. bellerica*, was examined using two modes of experimentation, i.e., co-incubation and pre-incubation in Ames assay. The antimutagenic effects were observed against direct-acting mutagens [sodium azide and 4-nitro-o-phenylenediamine (NPD)] and S9-dependent mutagen [2-aminofluorene (2AF)] in strains TA98 and TA100 of *Salmonella typhimurium.* Acetone extract showed maximum inhibition of 81.4% in pre-incubation mode of experimentation against 2AF at a concentration of 1x103 µg/0.1 ml. A bioactive acetone fraction was fractionated for the isolation of pure polyphenolic compounds. It was noticed that at the first step in the isolation 18.4% of ellagic and 9.2% of gallic acid was obtained. These compounds were compared with the standards using various spectroscopic techniques, viz., 1H-NMR, mass spectroscopy and IR. Further studies are in progress to isolate and to test the bioactivity of more fractions from the acetone extract.

Role of iNOS in alcohol-induced liver injury

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Alcohol consumption causes oxidative stress in liver and could be involved in triggering a vicious cycle of pathology. In support of this hypothesis, alcohol-induced liver injury is completely blocked in mice deficient in NADPH oxidase (p47phox knockout), indicating a key role of this enzyme in the progression of alcoholic liver injury. In addition to $O_2^{\cdot-}$, large amounts of NO· from iNOS are produced in liver when stimulated and could also play a role in radical-induced tissue damage. The aim of the current study was to test the hypothesis that oxidants from iNOS are involved in early alcohol-induced liver injury. Accordingly, wild-type and iNOS knockout mice were treated continuously with enteral ethanol for 4 weeks. Enteral alcohol feeding significantly increased liver to body weight ratios and serum ALT in wild-type mice, these effects were significantly blunted in iNOS knockout mice. Enteral ethanol also caused severe fatty accumulation, mild inflammation, and necrosis in the liver in wild-type mice, but did not cause pathology in iNOS knockout mice. Further, the formation of free radicals, 4-hydrox ynonenal and 3-nitrotyrosine protein adducts caused by alcohol was blocked completely in iNOS knockout mice. These data clearly show that knocking out iNOS in mice confers almost complete protection against the damaging effects of ethanol to liver, supporting the hypothesis that iNOS is required for the pathogenesis of early alcohol-induced hepatitis. These data also suggest a role for $ONOO^-$ in alcoholic liver injury.

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EMX-Herbal tea inhibits interleukin-8 release in alveolar epithelial cells

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The aim of this study was to determine whether EMX can inhibit the hydrogen peroxide (H_2O_2) and TNF-α-mediated release of the pro-inflammatory cytokine IL-8 in human alveolar epithelial cells (A549). There is recruitment of immune and inflammatory cells, which are activated to produce mediators of inflammation including oxidants and cytokines, such as the pro-inflammatory cytokine tumour necrosis factor-α (TNF-α). Oxidative stress has been implicated in the pathogenesis of several inflammatory disorders. One consequence of this process is to enhance the expression of both pro-inflammatory and protective antioxidant genes. Oxidants and inflammatory mediators such as tumour necrosis factor-α (TNF-α) activate transcription factors such as NF-κB and Activator Protein-1 (AP-1). Interleukin-8 (IL-8) is a ubiquitous inflammatory chemokine that mediates a multitude of inflammatory events. Hydrogen peroxide (H_2O_2) (100 μM) and TNF-α (10 ng/ml) imposed oxidative stress in A549 cells as shown by depletion of glutathione (GSH) concomitant with increased levels of oxidised glutathione (GSSG). EMX inhibited both H_2O_2 and TNF-α-mediated activation of NF-κB and AP-1. Both H_2O_2 and TNF-α significantly increased IL-8 release, which was inhibited by pre-

treatment of A549 cells with EMX compared to the control untreated cells. This study shows that EMX inhibits both the oxidant H_2O_2 and the pro-inflammatory mediator, TNF-α induced IL-8 and suggests a mechanism for the anti-inflammatory effects of EMX. We conclude that EMX may have therapeutic potential in neuroinfla-mmation and inflammatory diseases.

Macrophage reactive oxygen species induce LDL lipids peroxidation and atherosclerosis

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Oxidized LDL was shown to play a major role in macrophage foam cell formation, the hallmark of early atherosclerosis. To find out possible mechanisms which could be involved in macrophage-mediated oxidation of LDL, we have manipulated cellular oxygenases and macrophage antioxidants prior to analysis of their ability to oxidize LDL. Upon incubation of macrophages with LDL (in the presence of copper ions), activation of NADPH oxidase, followed by the production of superoxide anions and LDL oxidation, were demonstrated. When macrophages were exposed to oxidants such as angiotensin II or ferrous ions, cellular lipid peroxidation content was increased by 3 fold, in comparison with control cells. Incubation of these "oxidized macrophages" with LDL, resulted in lipoprotein oxidation (even in the absence of copper ions). Depletion of cellular glutathione by using BSO, an inhibitor of glutathione synthesis, significantly increased (by 3 fold) macrophage-mediated oxidation of LDL. Treatment of the atherosclerotic, apolipoprotein E-deficient (E^o) mice with selenium (1μg/mouse/wk, for a period of 3 months) resulted in a 44% inhibition in the ability of their peritoneal macrophages (MPM) to oxidize LDL. In parallel, a 30% reduction in the size of their atherosclerotic lesion was noted, in comparison to placebo-treated E^o mice. Some flavonoids increase cellular glutathion and some inhibit NADPH oxidase activity. Thus, we enriched macrophages with red wine derived quercetin, with licorice root derived glabridin or with pomegranate juice hydrolysable tannins. Cell-mediated oxidation of LDL by the flavonoids-enriched macrophage was inhibited by 50-90%, in comparison to control macrophages.

Finally, human serum paraoxonase (PON 1) activity is inversely related to the risk of atherosclerosis and PON 1 was shown to protect LDL from oxidation.

PON 1 was found capable of hydrolyzing and neutralizing oxidized lipids in oxidized LDL, as well as in atherosclerosis lesion. Nutritional antioxidants such as some flavonoids can preserve PON 1 activity and hence, stimulate its protection against the harmful atherogenic effects of lipid peroxides.

We conclude that intervention means to favorably affect the balance between macrophage oxygenases and the cellular antioxidative capacity, can protect LDL from oxidation and attenuate atherosclerosis.

Paraoxonase Protects against Lipid Peroxidation and Atherosclerosis

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Oxidative modification of low density lipoprotein (LDL) is a key event during early atherogenesis (1,2). Paraoxonase (PON 1) is associated in human serum with high density lipoprotein (HDL) and its arylesterase/paraoxonase activites were shown to be inversely related to the risk of coronary heart diseases, hypercholesterolemia and diabetes (3, 4). PON 1 exists in two polymorphic forms (5); one which differ in the amino acid at position 192 (glutamine or arginine, Q or R respectively) and the second one which differ in the amino acid at position 55 (methionine or leucine, M or L respectively).

PON 1 protects both LDL and HDL against oxidation (6, 7) and recently we have demonstrated increased protection of PON 1Q, in comparison to PON 1R against LDL oxidation (8). PON's ability to protect LDL against oxidation is paralleled by inactivation of the enzyme (9). Antioxidants protect LDL from oxidation and preserve PON 1 activity (9, 10). Dietary antioxidants such as polyphenols found in pomegranate juice, red wine or licorice can preserve or even increase PON 1 activity (10). It is not know whether PON 1 can act also on oxidized lipids in the atherosclerotic lesions. We have thus compared PON 1 isoforms Q and R for their effect on lipid peroxides in human coronary and carotid lesions, and their inactivation characteristics during PON 1 incubation with human atherosclerotic lesions. The mechanism for PON 1 action on lesion's lipid peroxides was laso analyzed.

After 24 hours of incubation with PON 1Q or PON 1R (10 arylesterase units/ ml), lipid peroxides content in both coronary

and carotid lesions was reduced up to 27% and 16% by PON 1Q and by PON 1R respectively. Further reduction in lipid peroxides was obtained upon lesions incubation with higher PON 1 activity (20 arylesterase units/ml of PON 1Q or PON 1R), with up to 44% and 26% decrement in lipid peroxides respectively.

PON 1Q was more potent than PON 1R not only in decreasing lesion's lipid peroxides content, but also in the time required to reach its maximal effect which was 2 hours and 5 hours respectively.

During incubation with coronary or carotid lesions PON 1Q and PON1R were inactivated and their arylesterase activities were decreased after 24 hours of incubation with the atherosclerotic lesions by 15% and 45% respectively.

To explore possible mechanisms for PON 1 action on lesion's lipid peroxides we have studied the effect of PON 1 on lesion as well as on purified cholesteryl linoleate hydroperoxides (CL-OOH) and hydroxides (CL-OH), and on linoleic acid hydroperoxides (L-OOH), using high pressure liquid chromatography (HPLC). Leision's CL-OOH and CL-OH were hydrolyzed by PON 1 to yield L-OOH and linoleic acid hydroxide (L-OH). Furthermore, lesion and pure L-OOH were reduced to yield L-OH. These results clearly indicate that PON 1 demonstrated an esterase and peroxidse-like activities.

PON1's free sulfhydryl group at cysteine – 284 was found to be important for its effect on reducing lipid peroxides content in oxidized lipoproteins and in human atherosclerotic lesion. Unlike the wild type recombinant PON 1 which decreased lesion's lipid peroxides by 35%, recombinant PON 1 muntants in which the PON 1 free sulfhydryl group was replaced, by site directed mutagenesis, with either alanine or serine, were no longer able to reucced lipid peroxides content in carotid lesion.

In summary, human serum paraoxonase (PON 1) may anti-atherogenicity may be related to its ability to hydrolyze and neutralize harmful cholesteryl ester hydroperoxides in lesion's lipoproteins and in arterial cells.

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Pomegranate juice as a major source for polyphenolic flavonoids and it is most potent antioxidant against LDL oxidation and atherosclerosis

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Atherosclerosis, the major cause of cardiovascular diseases is accelarated under oxidative stress (which also causes LDL oxidation). Thus, diet rich in antioxidants is recommended in order to attenuate LDL oxidation and atherosclerosis development.

The lipophylic antioxidants vitamin E and carotenoids however were found to be non effective in reducing atherosclerosis events in humans.

Polyphenolic flavonoids found in several fruits and vegetables in contrast, are mostly hydrophylics and they are potent antioxidants.

We thus analyzed and compared polyphenols content and ability to reduce LDL oxidation of several juices obtained from fruits. Pomegranate juice (Wonderful variety) was found to have 6.1 ± 0.4 mM of total polyphenols in comparison to Cranberry juice (4.5 ± 0.3), Red wine (4.2 ± 0.3) Bluberry Juice (3.7 ± 0.4), Green Tea (2.0 ± 0.3) and Orange Juice (1.8 ± 0.3).

Based on a similar volume content (0.5 microliter), the most potent antioxidant against copper ion – induced LDL oxidation was found to be pomegranate juice, followed by Cranberry juice, Red wine, Bluberry Juice, Green Tea, and Orange Juice, which were less potent than pomegranate juice by 17% , 25% , 25% , 35% and 55 % respectively.

Similar results were obtained when analyzing LDL oxidation by the TBARS method or by the lipid peroxides assay.

On a comparison based on a similar total ploy phenols content , pomegranate juice was again the most potent antioxidant against

LDL oxidation followed by Cranberry juice, Red wine, Bluberry Juice, Green Tea and Orange Juice, which were less potent than pomegranate juice by 10 % , 13 % , 30% , 40% and 70% respectively.

We thus conclude that pomegranate juice is a most potent anti-oxidant against LDL oxidation and this effect could be related to its high polyphenolic flavonoids content , as well as to the specific potent flavonoids present in pomegranate juice (tannins and anthocyanins) .

The potent anti-oxidative properties of pomegranate juice could explain its impressive anti- atherosclerotic effects as shown in mice and humans.

Onion juice polyphenols inhibits LDL oxidation: Stimulatory effect of juice storage and of the onion outer peel juice

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Onion is known to be a major source of the flavonol quercetin which is a potent antioxidant, found also in red wine and black tea. Onion quercetin is well absorbed, as shown by its accumulation in plasma and by its urinary secretion. Onion extract was shown to reduce arterial lesion size and plasma lipids levels in cholesterol - fed rabbits. As oxidation of LDL is thought to play a major role in the development of atherosclerosis, we have recently studied the effect of onion juice (extracted from onion by squeezing) on LDL oxidation. An onion dose-dependent inhibition of LDL oxidation was found with an IC –50 (the concentration required for the inhibition of LDL oxidation by 50%) of 4 µl/ml. Storage of the onion juice at room temperature (25°C) revealed that aging of the juice was associated with its improved capability to inhibit LDL oxidation. After 5 days of onion juice storage at room temperature, a 90% inhibition of LDL oxidation was obtained by 5 µl/ml of juice, in comparison to only 65% inhibition observed after one day of juice storage. In many fruits and vegetables, the flavonoids are located in the peel. We compared the juice (5 µl/ml) prepared from the various peels of the onion. The outer onion peel juice was found to be most potent with a 95% inhibition of LDL oxidation. The middle peel juice resulted in a 85% inhibition of LDL oxidation, and the inner peel was found to be less portent one with only a 35% inhibition in LDL oxidation. We thus conclude that onion juice possesses potent antioxidant activity against LDL oxidation which improves with juice storage time and it is mostly located in the outer peel which is rich in polyphenolic antioxidants. Onion juice may thus be considered anti-atherosclerotic agent.

Antioxidant activity of isolated polyphenols from a tropical pitcher plant

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Pitcher plant is one of the popular plants in tropical countries. The Nepenthese pitcher plant is the most widespread and common of all the tropical pitcher plants found in Singapore. Dried pitcher leaves were extracted with $CHCl_3$, EtOAc, MeOH and 80% MeOH. The extracts were used to measure the antioxidant activity. The 80% MeOH extract showed the highest scavenging activity. In addition, the 80% MeOH extract of dried pitcher leaves was purified by using phytochemical method and the purified polyphenol compounds were used for experiments. Free radical scavenging or antioxidant activity was examined using electron spin resonance (ESR) spectrometer.

Each of the purified polyphenol compounds formed phenoxyl radical spin adduct generated by HRP-H_2O_2- Etoposide assay and the antioxidant activity of each polyphenol compound was measured by ESR. This assay is based on the ability of one electron oxidation of Etoposide by horseradish peroxidase to produce phenoxyl radical intermediates. Trolox was used as a standard and compared with the purified compounds in terms of the antioxidant activities in Etoposide-H_2O_2-HRP assay.

These results demonstrated that 80% MeOH extract had high antioxidant activity. The phenolic compounds had moderate antioxidant activity and flavonoid compounds had high antioxidant activity.

Specific determination of 3-nitrotyrosine by HPLC and electrochemical detection using a combination of reduction and oxidation modes

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Several analytical procedures for 3-nitrotyrosine have previously been reported but the reported procedures lack in stability, specificity and/or specificity for the routine analysis of biological samples such as a hydrolysis sample of tissue protein. We describe here a novel analytical procedure by using inline carbon electrodes; a porous graphite electrode as a pre-electrolysis cell reducing 3-nitrotyrosine and a glassy carbon electrode for detection of the reduced 3-nitrotyr osine. The pre-electrolysis cell positioned up stream was a through-type electrode having an effective reduction capacity for 3-nitrotyrosi ne at –900mV vs. Au. The amperometric detection cell was set at +300 mV vs. Ag/AgCl, which is a considerably lower potential compared with 3-nitrotyrosine oxidation potential at +1100 mV vs. Ag/AgCl; this resulted in high specificity due to low applied potential and baseline resolution using a C18 column. The reduction effect of the pre-electrolysis cell was confirmed by the difference of a spectrogram profile obtained from 230 nm to 550 nm wavelength. The hydro-voltammogram obtained from reduced 3-nitrotyrosine might show that the nitro residue of 3-nitrotyrosine is reduced to an amino residue. This novel technique produced a lower detection limit at 10 fmol and was reproduced with over one thousand injections of biological samples without any maintenance of the detection system.

Intramitochondrial expression of catalase in *Drosophila melanogaster*: Effects on aging and stress resistance

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Intramitochondrial expression of catalase and a resulting decrease in hydrogen peroxide release from the mitochondria of transgenic *Drosophila melanogaster* have been described previously [1]. The purpose of this study was to characterize how this ectopic expression of catalase in mitochondria affects the aging process of the flies. The hypothesis was that introduction of catalase into the mitochondrial matrix would result in enhanced resistance to oxidative stress and retard the aging process. Experimental flies exhibited increases of up to 40% in their resistance to hydrogen peroxide and paraquat exposure, but their resistance to hyperoxia (100% oxygen) was only minimally enhanced. Catalase expression in mitochondria did not affect the aging process of unstressed flies, as indicated by their life spans and measurement of their metabolic rate and speed of walking. In comparison with catalase overexpression in the cytosolic compartment [2], intramitochondrial expression of catalase increased resistance to oxidative stress by a greater amount and over a wider range of oxidant concentrations. Overall, these results suggest that the localization and amount of antioxidant defenses influence the resistance to oxidative challenges, but the level of catalase activity does not affect the normal aging process. Future experiments will determine whether a balanced expression of Mn-SOD and catalase in the mitochondrial compartment leads to additional beneficial effects in *Drosophila*.

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The Lon protease preferentially degrades oxidized mitochondrial aconitase by an ATP-stimulated mechanism

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When incubated with bovine heart mitochondrial lysates, aconitase proteolysis gradually increased after *in vitro* treatment with H_2O_2 and then decreased with increasing peroxide concentrations. The proteolytic susceptibility of aconitase was not directly related to the decrease in its enzymatic activity. The enzyme responsible for this degradation is an ATP-stimulated serine protease, whose inhibitor/activator profile matched that of the mitochondrial Lon protease. For these studies, we fractionated the mitochondria matrix using size-exclusion and affinity chromatography. Maximal proteolytic activity was identified in a fraction corresponding to a molecular size matching that of Lon. After purification, silver staining on both native and SDS gels, as well Western blot analysis using polyclonal anti-Lon antibodies, revealed only one major band. The purified Lon protease was able to preferentially degrade oxidized aconitase in a process that was strongly stimulated by ATP, but not by non-hydrolyzable ATP analogues. Treatment of cells with morpholino oligonucleotides directed against the *lon* sequence produced significant down regulation of *lon* mRNA and Lon protein levels in WI-38 VA-13 cells. The *lon* deficient phenotype was characterized by a reduced ability to degrade both oxidized (carbonylated) and non-oxidized aconitase, which accumulated inside the cells. As a consequence these cells exhibited an initial increase in aconitase activity, followed by a sharp decline. Our data provide evidence that Lon is the major protease involved in the degradation of both normal and oxidized aconitase. Decreased levels of Lon are associated with impaired degradation and accumulation of aconitase.

Down regulation of the *Lon* protease causes impairment of mitochondrial morphology and function, and results in cell death

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We treated WI-38 VI-13human lung fibroblasts with morpholino oligonucleotides directed against the *lon* protease sequence, and we obtained a significant down-regulation of Lon expression. Four days after the treatment, the Lon depleted cells showed slow growth, increased cell death, and a ten-fold lower cell number than control oligonucleotide treated cells. When the anti-*lon* oligo treated cells were allowed to grow for another four days, the number of cells decreased even more between days four and six, and then remained stationary for the next two days, as the oligo morpholines were diluted in the cells. This phenotype can be partially rescued by *z*-VAD (50 μM), a general caspase inhibitor and by addition of the nucleotide uridine, which is required for cell division, and whose synthesis includes a mitochondrial enzyme.

We have also found alterations in a number of mitochondrial parameters such as respiration (both total aerobic cell respiration and complex I-IV activity), and mitochondrial transmembrane potential (Mitolight staining). Extensive morphological changes in mitochondria are seen by electron microscopy, such as mitochondria with no cristae, irregularly shaped mitochondria, giant mitochondria with the matrix space filled with electron-dense inclusions, and empty giant vacuoles.

We propose that cell death in the Lon deficient cells is due to ATP depletion caused by severe mitochondrial defects at a functional (loss of aerobic respiration and membrane potential) and morphological level, involving accumulation of oxidatively damaged proteins and possible loss of mtDNA genome integrity.

Modulation of Lon Protease activity and aconitase turnover with aging and oxidative stress

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Mitochondrial Lon protease mRNA levels are known to be decreased in aged murine muscle. We have recently proposed that this physiological down-regulation of the mitochondrial Lon protease is involved in the aging process. Since the Lon protease can preferentially degrade oxidized mitochondrial aconitase, its age-related decline might play a major role in the mitochondrial accumulation of oxidized and dysfunctional matrix enzymes.

Sod2+/- mice suffer a considerable amount of free radical damage and display a whole spectrum of disorders characteristic of pathological ageing, which renders them a good *in-vivo* model for free radical involvement in aging and disease.

The expression of Lon protease was lower in the old (27 months) *wt*, as well as young (3 to 6 months) and old (27 months) *Sod2+/-* mice, when compared with the young (3 to 6 months), *wt* animals. We found an overall increase in the level of oxidized proteins in old *wt* and *Sod2+/-* groups, with certain proteins being mostly carbonylated (80, 60 and 40 kDa bands). We identified the 80 kDa band and the 40 kDa band as being aconitase and an aconitase fragmentation product, with 3 times more carbonylated aconitase in the old *wt* and in the young *Sod2+/-*, and 4 times more in the old *Sod2+/-* group than in young, *wt* animals. The 60 kDa and 40 kDa bands followed different patterns, the former being increased only in old Sod+/- mice, while the latter actually decreased with aging only in wt animals. There was no significant difference in the level of mitochondrial aconitase as seen by immunoblotting.

In conclusion, we propose that declining Lon protease expression might be the cause for the accumulation of oxidized aconitase in old and oxidatively-challenged animals.

Short-term protein deficiency (PD) increases oxidative stress and induces emphysema in the lung of CD-1 mice

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Although cigarette smoke accounts for a large proportion of the cases of emphysema, little is known regarding the etiology of emphysema in non-smokers. In non-smokers with emphysema, diet may contribute to the pathogenesis of the disease. Previous studies have shown that animal models of chronic starvation result in emphysema. In this experiment, we hypothesized that dietary protein restriction would increase oxidative stress and consequently increase the susceptibility of the lung to develop emphysema. Weanling mice were fed a protein deficient diet (0.5% protein) and protein adequate diet (15% protein) for two weeks. PD resulted in a 20% weight loss, but food consumption was not different between groups. Our results indicate that reduced glutathione concentration was lower in the lungs of PD mice. Furthermore, myeloperoxidase was elevated indicating that the lungs of PD mice had higher neutrophil activation and consequently higher generation of reactive oxygen species. In addition, alpha-1 antiprotease was lower in PD animals suggesting that the lung of PD mice would have a higher rate of proteolysis. Contrary to our hypothesis, we found that oxidative defense enzymes (total superoxide dismutase and glutathione peroxidase) were also induced in the lungs of PD mice. These data suggest that acute PD results in a higher magnitude of oxidative stress in the lung and supports our hypothesis that PD may increase the susceptibility of the lung to develop emphysema. However, the role of dietary protein and the consequences of chronic oxidative stress in the pathogenesis of emphysema warrants further investigation.

Role of mitochondrial GSH in peroxynitrite mediate damage to mitochondria

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Peroxynitrite ($ONOO^-$) is a potent biological oxidant that can cause structural and functional alterations of mitochondrial proteins, leading to mitochondria dysfunction. GSH is considered a key cellular scavenger of $ONOO^-$. In order to assess the role of mitochondrial GSH in ONOO--mediated damage, isolated rat liver mitochondria were treated with 1-chloro-2,4-dinitrobenzene to deplete GSH. The effect of $ONOO^-$ on aconitase, a known mitochondrial target of $ONOO^-$, was studied in control and GSH depleted mitochondria. Exposure of mitochondria to $ONOO^-$ resulted in a dose-dependent inactivation of aconitase, accompanied by GSSG and GSNO formation. Unexpectedly, GSH depleted mitochondria were more protected from $ONOO^-$-induced aconitase inactivation. These findings suggests that the reaction between $ONOO^-$ and GSH generates reactive intermediates including GSSG, GSNO, thiyl radical (GS.) and $O_2\cdot^-$, that can damage aconitase With the help of an *in vitro* porcine heart aconitase system we investigated the important reaction products between GSH and $ONOO^-$ that can be responsible for aconitase inactivation. The results show GSNO and $O_2\cdot^-$ play a minor role compare to GS. and GSSG in $ONOO^-$-mediated aconitase inactivation. In conclusion, these results suggest that GS. and GSSG mainly contribute to the toxicity associated with peroxynitrite, and that GSH has under $ONOO^-$ stress, a prooxidant action.

Chlorination of Bacterial and neutrophil proteins during phagocytosis and killing *Staphylococcus aureus*

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Myeloperoxidase (MPO) is proposed to play a central role in bacterial killing by generating hypochlorous acid (HOCl) within neutrophil phagosomes. However, it has yet to be demonstrated that these inflammatory cells target HOCl against bacteria inside phagosomes. In this investigation we treated *Staphylococcus aureus* with varying concentrations of reagent HOCl and found that even at sub-lethal doses it converted some tyrosine residues in bacterial proteins to 3-chlorotyrosine and 3,5-dichlorotyrosine. To determine whether ingested bacteria were exposed to HOCl in neutrophil phagosomes, we prelabeled bacterial proteins with [13C6]-tyrosine and used gas chromatography with mass detection to identify the corresponding chlorinated isotopes after the bacteria had been phagocytosed. Chlorinated tyrosines were detected in bacterial proteins five minutes after phagocytosis and reached levels of about 2.5 moles per 1000 moles of tyrosine at 60 minutes. Inhibitor studies revealed that chlorination was dependent on MPO, and was confirmed when MPO-deficient cells were used. Chlorinated neutrophil proteins accounted for 94% of the total chlorinated tyrosine residues formed during phagocytosis. The extent of neutophil chlorination indicated that 70% of the H_2O_2 generated during phagocytosis was converted to HOCl. We conclude that HOCl is a major intracellular product of the respiratory burst. Although some reacts with the bacteria, the majority reacts with neutrophil components.

Decreased Bcl-2 expression and increased susceptibility to nitric oxide-induced apoptosis in G6PD-deficient human fore skin fibroblast cells

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Although glucose-6-phosphate dehydrogenase (G6PD) is a very important house keeping enzyme and its deficiency is the most common enzymopathy affecting over 200 million people world-wide, very little is known about how G6PD deficiency may affect cells other than red cells. Our laboratory has recently reported that human G6PD-deficient foreskin fibroblast cells (HFFs) exhibit retarded growth (Free Rad Biol Med 29: 156-1169, 2000) and are highly susceptible to nitric oxide (NO)-induced apoptosis (FEBS Letters 475: 257-262, 2000). To further delineate why G6PD-deficient HFFs are highly susceptible to NO-induced apoptosis, we investigated some of the proteins involved in the apoptotic cascade during NO-treatments. G6PD-deficient HFFs and normal HFFs were incubated with various concentrations of NO donor, sodium nitroprusside (SNP). P53, p21 and p73, which are known to stimulate apoptosis, were increased in a dose-dependent manner during NO-treatment. The increase of these proteins was more prominent in G6PD-deficient cells than in normal cells. More interestingly, the expression of Bcl-2 in G6PD-deficient HFFs was much lower than that in normal HFFs regardless whether NO was added. When G6PD-deficient HFFs were infected with the G6PD-expression retroviral vectors and started to over-express G6PD, the enhanced susceptibility to NO-induced apoptosis was corrected and the expression of Bcl-2 in these cells returned to normal. Taken together, these data indicate that enhanced susceptibility of G6PD-deficient HFFs to NO-induced apoptosis is closely related to the abnormal expression of certain proteins such as Bcl-2 in these cells.

Superoxide, Nitrogen Monoxide, and Carbon dioxide - Any Radicals?

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The question whether the self-decomposition of peroxynitrite yields ·OH radicals, and whether the reaction of $ONOO^-$ with CO_2 forms $CO_3^{\cdot -}$ radicals, is of great interest in biological processes. As transfers from the program of the OCC 2002 meeting, Koppenol argues in the title of his talk, as well as in his recent publications, against the radical model. He also raises doubt concerning the data presented by different research groups, claiming that his own and unique data is the correct one. We shall summarize below all the solid experimental data that supports the radical hypothesis, and point out the errors and artifacts in Koppenol's experiments which led him to his erroneous conclusions.

Antioxidant supplementation decreases F2-isoprostanes in plasma of passive smokers

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Background: Exposure to Environmental Tobacco Smoke (ETS) has been linked to increased risk of lung cancer and cardiovascular diseases in nonsmokers, and to several respiratory diseases in children (e.g., , such as asthma, bronchitis, and pneumonia). Current research suggests that some of these diseases are associated with elevated oxidative stress. .

Objective: We investigated the effect of antioxidant (AO) intervention was investigated on the lipid peroxidation biomarker F2-isoprostanes (F2IsoP), an index of oxidative stress, in plasma of passive smokers. In addition, we tested whether a combination of antioxidants AOs is more efficient in decreasing the oxidative stress biomarker F2IsoP than vitamin C alone.

Design: We measured F2-isoprostane F2IsoP concentrations in plasma samples of 66 passive smokers at baseline and after two months of daily intervention with antioxidants AOs or placebo. The study subjects (46 females, 20 males; mean age 46 ± 14, range 18-74 years) were randomized into one of three treatment groups: Vitamin C ((500mg), 'Cocktail' ((containing vit.amin C (500 mg), vit.amin E (400 -, 200 (-tocopherol, 50 -, 200 (- tocotrienol), - lipoic acid (100 mg)), and Placebo. Investigated confounders included dietary antioxidant AO intake, plasma baseline antioxidant AO levels, lipid and & total cholesterol profiles, transferrin saturation and C-reactive protein levels.

Results: Plasma F2IsoP F2-isoprostane concentrations of subjects in the vitamin C and cocktail group decreased significantly by 21.5 pmol/L ($p<0.005$) and 18.2 pmol/L ($p<0.01$) when compared to the placebo group (14.2%, 12.1%, respectively). This

multivariate analysis included adjustment for baseline F2IsoP F2-isoprostane levels, sex, body mass index, and race.

Conclusion: Daily AO intake decreases this oxidative stress biomarker in nonsmokers who are exposed to ETS. This finding might be of importance for the health of subjects who are involuntarily exposed to ETS, helping to prevent adverse health effects caused by ETS.

Genomic diversity in the human *SLC23A1* locus, which encodes the sodium dependent Vitamin C transporter

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The ubiquitously expressed integral membrane protein responsible for sodium-dependent Vitamin C uptake into cells is the sodium-dependent vitamin C transporter 2 (SVCT2) encoded by the *SLC23A1* gene. Due to its function as an antioxidant, vitamin C could be involved in the pathogenesis of cardiovascular diseases, diabetes, and stroke. We report here the annotation of the *SLC23A1* gene and specifically report common genetic variants and haplotypes, which could be informative as modifiers of monogenic or common diseases and aging. Re-sequence analysis was performed on 10.5 kb of different regions of the *SLC23A1* gene including in 96 anonymous controls (48 Caucasians and 48 African Americans): the open-reading frame, intron-exon borders, and the 3 prime un-translated region. We confirmed ten common (frequency > 1%) single nucleotide polymorphisms (SNPs) and two deletions of 5 and 16 basepairs, both within introns. Three SNPs were located in the coding region, all of which are synonymous. Frequencies varied slightly between the screened populations, but the differences are not statistically significant. The distribution of identified SNPs was determined in an additional subset of 24 Caucasians and 24 African Americans. On the basis of this latter group, common haplotypes have been inferred. Estimates of nucleotide diversity are π = 3.8 x 10-4 and θ = 1.9 x 10-4. The larger value of π indicates an excess of heterozygosity in *SLC23A1* that is significant by a statistical test of neutrality, Tajima's D statistic (D = 2.63, P < 0.05). Thus, we reject the null hypothesis that this locus evolved under conditions of neutrality and equilibrium. The presence of a

few relatively common SNPs within *SLC23A1* suggests that the observed haplotype variation could be the result of positive evolutionary pressure on the locus. These data imply a high degree of conservation in the gene encoding the sodium dependent Vitamin C transporter 2, indicating little tolerance for variation in *SLC23A1*. We believe that this gene possesses a physiologic importance and might be less constrained with respect to the generation of common variants within the coding region. Well-designed genetic association studies utilizing these and other markers in vitamin C transporter proteins could provide new insight into the role of vitamin C in disease states and perhaps, explain inter-individual differences in the pharmacokinetics of Vitamin C uptake.

Modulation of neutrophil apoptosis by lipid oxidation products

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Neutrophilic inflammation is generally accompanied by local oxidative stress, which can result in the oxidation of lipids to produce bioactive products. Among these products, α,β-unsaturated aldehydes such as acrolein and 4-hydroxynonenal (HNE) may mediate cellular responses to oxidative stress. Following our previous findings that acrolein can inhibit spontaneous apoptosis of human neutrophils, we further investigated effects of acrolein and HNE on neutrophil apoptosis. We observed that at low concentrations (1-10 μM) acrolein and especially HNE increased neutrophil apoptosis, shown by increased caspase-3 activation (measured by pro-caspase cleavage and caspase activity), annexin V binding (measured by flow cytometry), and cytochrome c release from mitochondria (measured by western blotting). Higher concentrations (>5 μM for acrolein, and >30 μM for HNE) displayed anti-apoptotic effects, with decreased caspase-3 activation, although cytochrome c release was further increased. Thus, it appears that both aldehydes can interfere with execution stages of apoptosis, despite the apparent activation of pro-apoptotic pathways. Collectively, both acrolein and HNE exhibit concentration-dependant biphasic effects on neutrophil apoptosis, with pro-apoptotic effects at lower concentrations and anti-apoptotic effects at higher concentrations despite increased cytochrome c release.

Sesamin, (+)-catechin, and butylated hydroxytoluene elevate vitamin E levels in male Sprague-Dawley rats

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Introduction. High blood concentrations of vitamin E have been associated with a reduced risk for degenerative diseases, such as cardiovascular disease (CVD) and cancer. α-Tocopherol (α-T), the predominant form of vitamin E in humans and animals, has been considered the most important tocopherol *in vivo* because it is retained in the body to a much higher extent than the other forms of the vitamin. On the other hand, plasma γ-tocopherol (γ-T), but not α-T concentrations have been shown to be inversely related to increased CVD morbidity and mortality. Also, γ-T may have as yet unknown specific functions in the body different from those of α-T. Therefore, γ-T deserves more scientific attention than it has been granted in the past. (1)

Human and animal diets contain countless phenolic substances which may interact with vitamin E absorption, metabolism and/or excretion and thus alter levels of a single "vitamer" or the net concentration of tocopherols. We employed the rat as a model in an attempt to screen some of the common phenolic compounds in the human and animal diets for their effects on α-T and γ-T concentrations in blood plasma, liver and lung tissue.

Materials and Methods. In all experiments, groups of male, 21-d old Sprague-Dawley rats were housed individually and fed basal diets (control) or identical diets fortified with a phenolic compound for 4 weeks. After the feeding period, the animals were sacrificed and blood plasma, liver and lung samples were collected

and analyzed for concentrations of α-T and γ-T by normal-phase high-performance liquid chromatography (HPLC).

Study I: Three groups of 10 rats were given an experimental diet, fortified with either sesamin or butylated hydroxytoluene (BHT) at a concentration of 4 g/kg, or the control diet (2).

Study II: Three groups of 8 rats were given an experimental diet, fortified with either (+)-catechin or butylated hydroxy-toluene (BHT) at a concentration of 2 g/kg, or the control diet.

Results. Sesamin did not change α-T values in any of the analyzed tissues, but increased γ-T ≈10-fold in plasma, ≈14-fold in the liver, and almost 17-fold in lung tissue ($P<0.001$ for all).

(+)-Catechin did not affect γ-T values, but increased α-T concentrations ≈2.5-fold in plasma, ≈3.5-fold in the liver, and ≈2.5-fold in the lungs ($P<0.0001$).

BHT elevated α-T concentrations ≈2.5-fold in plasma, ≈1.9-fold in the liver ($P<0.001$), and ≈3.7-fold in the lungs. No effect of BHT on γ-T concentrations was observed, at the 4 g/kg level, but at the lower dietary level, BHT lowered γ-T levels in plasma and liver tissue to about half of the control values ($P<0.05$).

Conclusions. The dietary phenolic substances sesamin, (+)-catechin and BHT elevate tocopherol levels in rats possibly due to interactions with absorption, metabolism, and/or excretion.

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Flow chart of biochemical interactions causing human aging

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The many observable signs and symptoms of human senescence have been hypothesized by various researchers to result from several primary causes. Close inspection of the biochemical pathways associated with each of the hypothesized causes of human senescence shows interactions and feedback among them.

As an aid to keeping track of the many interactions, a flow chart is presented, which includes:

- Glycation of long-lived proteins and nuclear DNA.
- Accumulation of mutations in the mitochondrial genomes of postmitotic cells.
- Increasing acetylation of histones opens heterochromatin, permitting inappropriate expression of nuclear genes.
- Accumulation of lipofuscin in postmitotic cells.
- Increased redox poise alters signaling and enzyme activities.
- Redox damage and crosslinking of long-lived macromolecules in postmitotic cells and extracellular matrix.
- Telomere shortening induces altered phenotype and halts cell division in some cells.
- Cell death lead to tissue wasting, neurodegeneration, and organ malfunction.
- Alterations in neuroendocrine and immune systems.
- Slowing repair & turnover of macromolecules and organelles.
- Export of toxic reactive species and cytokines from senescent cells.
- Induction of cancer.

This flow chart will be maintained on the Web as a reference to researchers, and will be updated as new information comes to light. [www.LegendaryPharma.com/senescence.html#Mechanisms]

Dopamine oxidation in cell culture systems

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The oxidation of intracellular dopamine (DA) is thought to be the process responsible for the selective neurodegeneration of pigmented dopaminergic neurons in the substantia nigra in Parkinson's disease (PD). The mechanisms of dopamine-induced neurotoxicity are believed to involve the generation of superoxide ($O_2\cdot^-$), hydrogen peroxide, dopamine-semiquinone, and dopaminochrome (precursor of neuromelanin). However data on the toxicity of DA, typically obtained in cell culture systems, do often not take into account that DA is oxidized in the cell culture media. Thus, it becomes important for the elucidation of possible mechanisms involved in dopamine-mediated toxicity to differentiate between the effects of DA and the effects of its extracellular oxidation products.

The role of DA oxidation in a variety of cell culture media was measured. The results show the importance of low molecular weight antioxidants such as GSH and L-Cysteine in the cell culture media with regard to the stability of DA. Furthermore, the findings emphasize the role of metal chelators such as transferrin to prevent extracellular oxidation of dopamine *in vitro*. Taken together it becomes clear that these factors need to be considered when interpreting experiments using DA in cell culture.

Vitamin C protects from cell death induced by oxidative stress in glutathione-depleted human leukemia cells

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Vitamin C is a well-known antioxidant whose precise role in protecting cells from oxidative challenge is uncertain. *In vitro* results have been confounded by pro-oxidant effects of ascorbic acid and an overlapping role of glutathione. We used HL-60 cells as a model to determine the precise and independent role of vitamin C in cellular protection against cell death induced by oxidative stress. HL-60 cells do not depend on glutathione to transport or reduce dehydroascorbic acid. Depletion of glutathione rendered the HL-60 cells highly sensitive to cell death induced by H_2O_2, an effect that was not mediated by changes in the activities of glutathione reductase, glutathione peroxidase, catalase or superoxide dismutase. The increased sensitivity to oxidative stress was largely reversed when glutathione-depleted cells were preloaded with ascorbic acid by exposure to dehydroascorbic acid. Resistance to H_2O_2 treatment in cells loaded with vitamin C was accompanied by intracellular consumption of ascorbic acid, generation of dehydroascorbic acid, and a decrease in the cellular content of reactive oxygen species. Some of the dehydroascorbic acid generated was exported out of the cells via the glucose transporters. Our data indicate that vitamin C is an important independent antioxidant in protecting cells against death from oxidative stress.

Antioxidants attenuate staurosporine-induced apoptosis by inhibiting the activation of caspase cascade

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The pathogenesis of neurodegenerative disorders is frequently associated with programmed cell death or apoptosis. The biochemical events of apoptosis are executed by a large group of cysteine proteases called caspases. Among this class of proteins, caspase-3 is known to be a downstream effector in triggering the process of apoptosis. Oxidative stress induced by ROS may cause the activation of caspase-3 through the induction of cytochrome c from mitochondria and may subsequently induce downstream cascades. Flavonoids, certain estrogen derivatives, and phytoestrogens have been demonstrated to act as suppressors of caspase activity [1,2]. Recently, we have found that certain phenols, aromatic amines and imines, are highly potent cytoprotective antioxidants, which act as direct ROS scavengers [3]. Here we investigate the interaction of these antioxidants with the activation of caspase-3 and the involved mechanisms. Clonal hippocampal HT22 cells are pretreated with phenothiazine, phenoxazine and 2,4,6-trimethyl phenol (12 h following 50-100 nM staurosporine exposure) and the protein levels of activated caspase-3 are analyzed by Western Blotting. The goal of our study is to analyze whether potent antioxidant compounds have an additional protective activity by blocking the intracellular caspase network activated by triggers of apoptosis.

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Superoxide generation into the intermembrane space by the respiratory chain and diffusion to the cytoplasm through VDAC in the outer mitochondrial membrane

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The mitochondrial electron transport chain is the major cellular source of superoxide anion ($O_2{\cdot}^-$), largely originating from autoxidation of ubisemiquinone. $O_2{\cdot}^-$, produced in this manner, can be vectorially released towards the mitochondrial matrix or the intermembrane space, for ubisemiquinone formation occurs on the inner membrane both near the matrix (Q_I site) and the intermembrane space (Q_O site). $O_2{\cdot}^-$ release into the intermembrane space was explored using EPR in conjunction with spin traps. EPR analysis of isolated heart mitochondria revealed the formation of a DMPO-OH adduct originating from the spontaneous decay of a DMPO-superoxide adduct. The DMPO-OH signal generated by heart mitochondria was abolished by addition of exogenous Mn-SOD and ferricytochrome *c*. Because neither Mn-SOD nor ferricytochrome *c* can cross the outer mitochondrial membrane, this suggests that DMPO reacted with $O_2{\cdot}^-$ that had diffused across the porous outer membrane. The porous nature of the outer mitochondrial membrane is due to a voltage dependent anion channel (VDAC) that is 2-4 nm wide and more selective for anions than cations. VDAC can be forced into a closed conformation by addition of polyvalent anions like dextran sulfate (when a membrane potential is present). To determine whether VDAC was responsible for $O_2{\cdot}^-$ diffusion across the outer membrane, isolated heart mitochondria were treated with dextran sulfate (MW = 8 kDa). Treated heart mitochondria showed a 25% decrease in $O_2{\cdot}^-$ diffusion. Bovine serum albumin, (BSA; MW = 67 kDa) a negatively

charged membrane-impermeable protein, was added to mitochondria to generate a membrane potential along the outer membrane. In the presence of BSA, dextran sulfate decreased $O_2\cdot^-$ diffusion by 80% in isolated heart mitochondria. The fact that $O_2\cdot^-$ diffusion across the outer membrane could be modulated by changes in voltage, confirmed VDAC as the primary channel responsible for $O_2\cdot^-$ diffusion. Due to the presence of VDAC in the outer membrane, a large portion of $O_2\cdot^-$ generated by the mitochondrial respiratory chain may end up in the cytoplasm. Mitochondria must therefore be viewed as cytoplasmic sources of $O_2\cdot^-$, as well as H_2O_2.

Differential expression of *DSCR1 (Adapt78)* gene isoforms 1 & 4 in Alzheimer disease

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DSCR1 (Adapt78) was identified by our laboratory, during differential display studies, as a gene that was strongly upregulated in mammalian cells during transient adaptation to oxidative stress. *DSCR1 (Adapt78)* lies within the Down's syndrome candidate region of chromosome 21, a segment of the genome linked with several disorders associated with oxidative stress. *DSCR1 (Adapt78)* is expressed in several human tissues, with particularly significant levels in the brain; including the cerebral cortex, hippocampus, substantia nigra, thalamus, and medulla oblongata. The *DSCR1 (Adapt78)* protein product, calcipressin 1, has recently been identified as an inhibitor of calcineurin, which is the major serine threonine phosphatase in brain. We have previously proposed a role for chronic expression of *DSCR1 (Adapt78)* in Alzheimer's disease and have shown mRNA levels to be induced approximately two fold in brain autopsy samples from patients who died with Alzheimer disease and Down Syndrome. The *DSCR1 (Adapt78)* gene contains seven exons which are alternatively spliced. Two isoforms have been found to be expressed in brain tissues, isoform 1 - consisting of exons 1,5,6,7; and isoform 4 - consisting of exons 4,5,6,7. We are exploring the expression of both isoforms in various brain cell types in normal and Alzheimer disease postmortem samples, using the combined technique of immunohistochemistry and in situ hybridization.

Cigarette smoke induced nuclear factor-κB activation and apoptosis inhibition in human lymphocytes is mediated by nitric oxide and peroxynitrite

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Cigarette Smoke (CS) is the major risk factor for developing inflammatory diseases of the lung and respiratory tract, such as emphysema and chronic bronchitis. In addition, CS exposure in children of smoking parents has been associated with severe Asthma, in a mechanism which is still poorly understood. NF-κB is a dimeric transcription factor that can rapidly activate tissue-specific expression of genes involved in inflammatory and immune response. Recent evidances suggest a role for oxidative and nitrosative stress in the regulation of NF-κB activation. In the present study we hypothesized the ability of CS to activate NF-κB in human peripheral blood lymphoytes (PBL's). Using a barrometer controled, nitrite concentration monitored vacuum derived smoking apparatus, we were able to demonstrate the translocation of the p65 subunit of NF-κB from the cytoplasm to the nucleus at low exposure (negative pressure 50 mmHg, $[NO_2]$=4.8 ± 2.8 μM) but not at high exposure (neg. pressure 200 mmHg, $[NO_2]$= 21.4 ± 6.6 μM). This translocation was mediated by phosphorylation and consequent degradation of the inhibitory protein, I_B_. CS induced NF-κB translocation was associated with a 42% decrease in intracellular GSH/GSSG ratio, which was almost completely reversible within 180 minutes. Inhibition of spontaneous apoptosis was demonstrated in those mildly exposed lymphocytes by FACS analysis using AnnexinV-FITC. Both nitric oxide and peroxynitrite donors were able to mimic the effects of CS on NF-κB activation and apoptosis inhibition. Ebselen, a potent RNS

inhibitor, decreased those effects of CS. Currently we are studying the bio-medical implications of such CS induced NF-κB activation, its role in gene expression and production of inflammatory cytokines, and other aspects of initiation and propagation of inflammatory processes.

Effect of Japanese herbal medicine (Toki-Shakuyaku-San) on DNA damage in aged rodent brain and C6 glia cell death

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Japanese herbal medicine, Toki-Shakuyaku-San (Tsumura & Co., Tokyo) has free radical scavenging activities against superoxide, hydroxyl and 1,1-diphenyl-2-picryl-hydrazyl radicals. It has been shown to decrease elevated lipid peroxide level in aged rat brain. It has an enhancing activity on cholinergic neuronal functions in the striatum of aged rat. DNA damage has been evaluated by the assay of 8-hydroxy-2'-deoxyguanosine formation, and the damage in rodent brain was found to increase with age. Cell death is related with reactive oxygen free radicals. In the present study Toki-Shakuyaku-San was examined to affect DNA damage in brain with age and C6 glia cell death. 8-Hydroxy-2'-deoxyguanosine was assayed using high performance liquid chromatography and cell death was evaluated by induction with glutamate using lactate dehydrogenase assay. Senile accelerated mice (SAMP8) of 3-4 and 7-12 months old were used for experiment. The following results were obtained; (1) DNA damages in brain and liver of SAMP8 at 3-4 months old were accelerated compared to those of control SAMR1 mice, and Toki-Shakutyaku-San decreased the DNA damage in brain of SAMP8. (2) DNA damage was elevated in aged rat brain of 24 months old, and Toki -Shakuyaku-San lowered it. (3) Toki-Shakuyaku-San inhibited glutamate-induced C6 glia cell death. These results suggest that inhibitory effects by Toki-Shakuyaku-San may be due to its free radical scavenging activities. In conclusion, Toki-Shakuyaku -San may protect or may delay to have a lapse of memory and dementia.

Prevention of zonisamide on oxidized proteins formed in synaptosomal membrane during iron-induced epileptogenesis of rats

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Free radicals induce lipid peroxidation, and oxidize DNA and protein. Previously we found increase in levels of lipid peroxide and 8-hydroxy-deoxyguanosine (8-OHdG) in rat brain with iron-induced epileptogenesis formation of acute stage. Zonisamide (ZNS) has free radical scavenging activity, and it inhibited the reaction of lipid peroxidation and the increase in 8-OHdG level of rat brain with iron-induced epileptogenesis formed of acute stage. In the present study we examined oxidized protein formation in brain with iron-induced epileptogenesis of rat, and effect of zonisamide on it was also studied. Dainippon Pharmaceutical Co., LTD., Osaka, supplied ZNS. Male Wistar rats weighing 200g were injected with ZNS (100mg/kg) intraperitoneally 30 min before injection of 0.1M $FeCl_3$ into the left sensory motor cortex. After injection of the iron solution, the ipsilateral cortex was excised 30 min after injection and synaptosomal membrane was obtained using density gradient centrifugation method. Oxidized protein was evaluated by assay of carbonyl protein and of alterations in electron spin resonance spectral parameter of MAL-6-labeled membranes, the ratio of W/S. The level of carbonyl compound was increased and the ratio of W/S was decreased in the ipsilateral synaptosomal cortex 30 min after iron solution injection into the left sensory motor cortex of rats. ZNS in dose of 100mg/kg reduced the elevation of carbonyl compound and the fall of ratio of W/S. The decrease in the ratio of W/S means formation of oxidized protein. ZNS inhibited changes in carbonyl compound level and the ratio of W/S in the ipsilateral synaptosomal cortex by iron solution injection. These

protective effects of ZNS may be due to its free radical scavenging activity. Previously we observed prevention of ZNS on the elevation of lipid peroxidation in the ipsilateral cortex and 8-OHdG formation in the cerebrum during iron-induced epilepto-genesis formation in rats. In consideration of these results, ZNS may be effective for prevention against neurological diseases associated with free radicals.

Ebselen:
A drug targeting thioredoxin and thioredoxin reductase

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Ebselen (2-phenyl-1,2 benzisoselenazol-3(2H)-one) is a glutathione peroxidase mimic used in clinical trials against stroke. Human and bovine TrxR catalysed the reduction of ebselen to ebselen selenol by NADPH with a Km value of 2.5 µM and a kcat of 588 min-1. Addition of thioredoxin (Trx) stimulated the TrxR catalyzed reduction of ebselen several-fold. This is due to a very fast oxidation of *reduced* Trx by ebselen with a rate constant in excess of 2×10^7 $M^{-1}s^{-1}$. This rate is orders of magnitude faster than the reaction of dithiol Trx with insulin disulfides. Ebselen competed with disulfide substrates for reduction by Trx and acted as an inhibitor of protein disulfide reduction by the thioredoxin system. The inherent hydrogen peroxide reductase activity of mammalian TrxR dependent on its active site selenocysteine residue was stimulated 15-fold by 2 µM ebselen and 30-fold in the additional presence of 5 µM Trx. Furthermore, the apparent Km of TrxR for hydrogen peroxide was lowered 25-fold to about 100 µM. Our results demonstrate that ebselen is a TrxR peroxidase which in the presence of Trx acted as a mimic of a peroxiredoxin. The activity with TrxR and oxidation of reduced Trx offer mechanistic explanations for the *in vivo* effects of ebselen as an antioxidant and antiinflammatory agent. The mechanism of action of ebselen may be predominantly via the thioredoxin system rather than via glutathione.

Nanotechnology to characterize inflammatory responses at the arterial bifurcations

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Introduction: Atherosclerotic lesions are prevalent at the curvatures and lateral walls of vascular branching points. The emerging nanotechnology facilitates the elucidation of the mechanisms by which shear stress regulates monocyte chemoattractant protein-1 (MCP-1) expression. Pulsatile flow characterized as unidirectional net forward flow downregulates MCP-1 expression, whereas oscillatory flow characterized as zero net forward flow upregulates MCP-1 expression. **Methods:** Micro sensors, comparable to the dimension of an elongated bovine aortic endothelial cell (BAEC), were used to measure real-time shear stress acting on ECs. BAECs, which were treated with oxidized low density lipid protein (ox-LDL), were subjected to three known vascular flow conditions at 60 cycles/min: (1) high shear stress upstroke slope ($\partial\tau/\partial t$ = 293 dynes/cm^{2}sec, time-averaged shear stress (τave) = 30 dynes/cm^2), (2) low shear stress upstroke ($\partial\tau/\partial t$ = 71 dynes/cm2sec) with identical τave, and (3) reversing oscillating flow (0 $\pm$ 3.1 dynes/cm^{2}sec). Reverse transcription-polymerase chain reactions (RT-PCR) were performed for MCP-1 mRNA expression. PCR products were analyzed by agarose gel electrophoresis containing SYBR Gold® nucleic acid gel stain and by densitometric scanning of the DNA bands. The intensities of all bands were normalized to that of GAPDH and analyzed under identical conditions. **Results:** High $\partial\tau/\partial t$ pulsatile flow downregulated MCP-1 expression by 33 $\pm$ 8 % and low $\partial\tau/\partial t$ by 15 $\pm$ 4 %, whereas oscillating flow upregulated MCP-1 by 13 $\pm$ 5 % (Figure 1). These results suggest that both the upstroke slopes of pulsatile shear stress and oscillation regulated the expression of a potent monocyte chemoattractant at bifurcations.

Kinetic comparison of anthocyanin reactivity toward reactive oxygen and nitrogen species using capillary zone electrophoresis

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Recently, we have established capillary zone electrophoretic separation of anthocyanins and showed that this method was useful for analyzing food materials containing anthocyanins such as blueberry. In the present study, we evaluated the reactivity of anthocyanins toward reactive oxygen and nitrogen species such as H_2O_2 and peroxynitrite using this method. From the comparison of each reactant concentration needed for 30% decrease of A520nm of the bilberry extract, the reactivity of anthocyanin toward active oxygen species was determined in the following order: H_2O_2 > t-BuOOH >AAPH (EC30:: 5mM for H_2O_2, 50mM for t-BuOOH and 180mM for AAPH radical). The reactivity of each anthocyanins toward reactive oxygen species was determined from the decrease of peak height of each anthocyanins on electropho-retogram. It was found that the reactivity of anthocyanin toward AAPH radical was governed primary by anthocyanidin (the aglycon of the anthocyanin) structure, it was clearly shown that delphinidins were more reactive than cyanidins in any glycosides. Further, the methylation of the hydroxyl group on anthocyanidin B ring reduced the reactivity of anthocyanin toward AAPH radicals. The reactivity of anthocyanins toward H_2O_2 was completely different from that toward AAPH radicals. The reactivity of anthocyanin toward H_2O_2 was not significantly affected either by the aglycon structure or by the conjugated sugar type, and was several times higher than (+)-catechin that was measured as a reference antioxidant. The reactivity of anthocyanins toward t-BuOOH was essentially the same as that toward H2O2 [3]. Since methylation of the hydroxyl substituent on B ring reduced the reactivity toward

AAPH radicals, it was suggested that AAPH radicals reacted to anthocyanins through hydrogen atom abstraction from the hydroxyl group(s) on B ring as same as to other flavonoids.Å@On the other hand, H_2O_2 attacksÅ@2,3-double bond of anthocyanidin C ring to cleave it [4]. It was thus suggested that anthocyanin functions as a strong hydroperoxide scavenger.

When the reaction of anthocyanin was studied to reactive nitrogen species, the reactivity of anthocyanin can be listed in the following order: peroxynitrite>NO (EC30:: 3mM for NO and 1.3mM for peroxynitrite),. The reaction of anthocyanins with NO was markedly enhanced under aerobic conditions probably because peroxynitrite was generated secondary. Thus the reaction was evaluated under strictly anaerobic condition. CZE study revealed that delphinidins carrying three hydroxyl groups on B ring of the aglycon were the most reactive toward NO and peroxynitrite among 12 anthocyanins determined in wild berry extract. Interestingly, the reactivity of delphinidin 3-glycoside toward peroxynitrite was approximately two times greater than that toward NO, whereas all other anthocyanins including cyanidin carrying two hydroxyl groups on B ring of the aglycon were less reactive toward peroxynitrite than NO.

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Aging versus reliability: Free-radical and antioxidant modulations of the genetic melodies

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The goal of this study was to understand why, despite complexity of mechanisms, aging is governed by the relatively simple quantitative laws. The concept, that all molecular biological constructions perform their functions with the genetically limited reliability, served as the methodology of this study. The universal laws of aging, such as Gompertzian growth of mortality and correlation of longevity with the species-specific resting metabolism, are easily and naturally explained on this basis. The random malfunctions of mitochondrial respiratory chains are of the first importance since they produce toxic superoxide radicals. Mitochondria become intensive generators of superoxide radicals after transient anoxia/ischaemia conditions. This loss of control for the electron flow is caused by increase in the membrane lipid fluidity. Since all defense systems, amongst them - mtSOD defense and DNA-repair enzymes, operate with limited reliability, stochastic free-radical damages accumulate in DNA up to the threshold dysfunction level, resulting in the apoptotic loss of cells. The replicative capacity of diploid cells is also limited owing to the gradual loss of telomeres. Hence, the limited reliability of the mitochondrial enzyme machinery, along with the telomere marginotomy, causes the loss of functionally competent cells. We estimated that the longevity of human brain could reach 250 years, should the reliability of mtSOD-defense be absolutely perfect. the reliability of mtSOD-defense be absolutely perfect. Thus, the concept of limited reliability of biological systems pieces together the free-radical theory of aging and the theory of telomere marginotomy.

Metabolism of vitamin E: A single dose-response study

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Plasma levels of α-tocopherol (α-T) do not adequately represent vitamin E status, and other measures are required to identify the true extent of vitamin E's involvement in disease states.

The study aims to improve the understanding of vitamin E status in healthy individuals by determining urinary metabolite dose-response relationships with vitamin E in blood components.

Nineteen healthy volunteers were assigned a vitamin E dose (either 134, 201, 402 or 805 mg *RRR*-α-tocopheryl acetate). Twenty-four hour urine collections were taken 2 days prior to (baseline) and 3 days following ingestion of the vitamin E capsule. A fasting blood sample was also taken 24 h before and after the dose.

A single dose of α-T produced a transient response in the excretion of α-CEHC. A large inter-individual variation in baseline α-CEHC and response to α-T dose was observed, with no clear dose-response. The percentage of α-T dose excreted as α-CEHC was very low (<1%). Doses 134, 201 and 402mg produced a maximal α-CEHC excretion on day 4, whereas the 805mg dose produced a maximal increase on day 3. Plasma α-T increased and γ-T decreased following each dose. γ-CEHC excretion also increased, % change was significantly greater ($p = <0.05$) with 805mg α-T. The low percentage dose excreted as α-CEHC is presumably due to the selective retention of α-T by the α-tocopherol transfer protein, which may become saturated at higher doses (maximal excretion of α-CEHC on day 3 after 805mg dose). The decrease in plasma γ-T and increase in γ-

CEHC following α-T supplementation may also be due to selective retention of α-T in plasma. Due to the large inter-individual variation we are unable to conclude whether there is a dose-response to α-T. Future studies aim to investigate multiple dosing and the variation in individual response.

Cholesterol oxidation products and TGFβ1 expression

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Oxidized LDL are known to affect various cellular processes by modulating molecular transduction pathways and signaling nuclear transcription. In particular, the proatherosclerotic effect of oxLDL is well recognised despite the underlying molecular mechanisms of action are still largely undefined. Among the various oxidative breakdown products of LDL lipid moiety, aldehydes have been so far the most characterised for their potential contribution to free radical phatobiology. The most representative one, 4-hydroxy-2,3-nonenal (HNE), at doses compatible to those detectable " in vivo" (1-10 μM), induces the expression of a number of genes including those coding for collagen type I and TGFβ1, most likely through up-regulation of AP-1 transcription factor. LDL contain numerous other lipid oxidation products aside from aldehydes, in particular oxysterols, i.e. 27-carbon products of cholesterol oxidation, and core aldehydes, derive from cholesterol esters oxidation, which may become quantitatively important in hypercholesterolemic subjects. Recently, incubating murine or human macrophages with a biologically representative mixture of oxysterol (10-30 μM) or with 9-oxononanoyl cholesterol (1-20 μM), a major cholesterol ester core aldehyde, a consistent and significant enhancement of both expression and synthesis of the fibrogenic cytokine TGFβ1 was demonstrated. Identical concentration of unoxidized cholesterol or cholesteryl linoleate did not show ability to modulate the

cytokine expression and synthesis. All toghether these findings point to a primary role for LDL lipid oxidation products in stimulating fibrogenic cytokine as an integral step in the atherogenic process. TGFβ1 up-regulation in early atherosclerotic lesions suggests a specific mechanism by which the formation and elaboration of a fibrotic plaque can take place.

Nutraceuticals protect fibroblasts during oxidative stress and aging

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A number of studies revealed the role of oxidative stress during the aging process. An age-related decline in the antioxidative defense, including declined antioxidant levels, the increase of markers of lipid peroxidation, of DNA and protein oxidation and the accumulation of highly oxidized lipofuscin is well established. These data could be achieved by investigating cellular aging models, such as proliferative and post-proliferative cellular senescence models, or due to the investigation of animals and human beings.

Due to the increasing interest in the age-related changes in the metabolism the question rises, whether we can prevent or at least slow down these processes. Since the main intracellular metabolic changes occur in the mitochondrial energy metabolism and the antioxidant defense system, we tested the effect of several nutraceuticals on the stabilization of parameters of these metabolic pathways.

We tested creatine as an stabilisator of the energy metabolism, carnitine and acetyl-L-carnitine, as well as the precursor molecule γ-butyrobetaine, all enhancers of the mitochondrial energy transport and R-lipoic acid and ubichinone as antioxidant molecules. These components were tested as single compounds or in various combinations. Several investigations were performed in the presence with PBN, a well known spin trap. As parameters we choose viability and growth characteristics of the cells, determination of the adenine nucleotide content, the TPP+

uptake, the glutathione status, lipid peroxidation or the cellular autofluorescence.

We used an MRC-5 fibroblast model of proliferative senescence and the same fibroblasts immortalized with an SV40 virus to test the effect of oxidative stress on these cells. The predominant effect was measured after addition of R-lipoic acid.

Antioxidant capacity of apple polyphenols

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Apples are considered one of the most important sources of flavonoids and other polyphenols in the human diet, together with wine, tea and onions. We studied the antioxidant capacity of the most common polyphenols in apples by their ferric reducing ability potential (FRAP) and oxygen radical absorbing capacity (ORAC). FRAP, ORAC and polyphenol concentrations (1.5–12.5 μM) were linearly correlated, with flavonols and flavanols being the most effective. FRAP and ORAC results, respectively, showed that 1 μM trolox (Tx) was equivalent to 0.3 and 0.3 μM quercetin, 0.7 and 0.3 μM rutin, 0.6 and 0.4 μM epicatechin, 0.6 and 0.4 μM catechin, 1.2 and 0.5 μM chlorogenic acid, 0.8 and 0.5 μM phloretin, and 8.3 and 0.5 μM phloridzin, the latter two apple-specific dihydrochalcones.

Aqueous extracts from different varieties of apples were characterized for antioxidant capacity and polyphenolic content. Extracts from whole (flesh and skin) Red Delicious apples (RD) had the highest antioxidant capacity (2877±91 and 3052±89 μM Tx equivalents for FRAP and ORAC, respectively) compared to Granny Smith (1902±70 and 1635±22 μM) and Fuji (1595±90 and 1697±178 μM). RD extracts also contained the highest levels of total phenolics (357±6 mg/l), total catechins (30.4±1.0 mg/l), and phloridzin (5.3±0.6 mg/l). The addition of RD extracts to human plasma in the range of 2–20 mg/ml significantly increased plasma FRAP in a linear manner, from 452±17 to 688±20 μM Tx equivalents, but did not affect plasma ORAC. In plasma containing 4–8 mg/ml of RD extract, AAPH-induced oxidation of endogenous urate was delayed, while ascorbate

oxidation was not affected. Preliminary *in vivo* results indicate that FRAP and ORAC of human plasma transiently increase after the consumption of apples. These results could be explained only partially by the increase in ascorbate concentration, suggesting that polyphenols in apples contribute to increased antioxidant capacity of plasma in humans.

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Experimental evidence supporting formation of radical intermediates by O-O bond homolysis during ONOOH decomposition

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The issue of whether or not significant amounts of $OH\cdot$ and $NO_2\cdot$ radicals are formed during ONOOH decomposition has been actively debated for almost a decade. Current discussion is focused upon the accuracy of data that are critical to supporting the radical hypothesis, with different laboratories reporting contradictory observations. Recent unpublished results from our own laboratories appear to be fully compatible with the radical hypothesis. Specifically: (1) an extensive study of the pressure dependence of ONOO decomposition and first-order oxidation reactions indicates a common volume of activation of +9.7 ± 1.4 cm3/mol. This value is consistent with O-O bond cleavage to yield neutral fragments, but difficult to rationalize in terms of the alternative suggested mechanism involving intramolecular isomerization as an activation mechanism; (2) the disputed rate constant for ONOO- dissociation into $O_2\cdot$ - and $NO\cdot$, originally reported by Merenyi and Lind in support of the radical hypothesis, has been confirmed by an entirely different method of measurement. This rate constant has been a focal point of scrutiny because it is crucial to accurate kinetic modeling of the decomposition kinetics by the radical pathway and to thermodynamic arguments for and against that hypothesis; (3) contrary to a recently published report, we find that the NO_2- yields obtained upon decomposing peroxynitrite in weakly alkaline solutions is accurately predicted by the radical mechanism over the entire experimentally accessible range. Furthermore, kinetic analyses of peroxynitrite decomposition at concentrations up to 25 mM are inconsistent on several grounds with the proposed alternative mechanism for NO_2- formation involving direct bimolecular reaction of ONOOH with $ONOO^-$.

The level of vitamin E and C at patients with age – related cataract

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The level of vitamin E and C in the blood plasma of the patients with age-related cataract, observed in cataract department of Filatov Research Eye Institute was investigated. The patients with nuclear, mixed and especially cortical forms of cataract had significant decrease in vitamin E (up to 87%, >.05, 80 %, < .05 and 78%, < .01). During subcapsular cataract the level of vitamin E and C is decreased twice, (<.01). By maturation of cataract the decrease of vitamin E concentration is growing in comparison with a transparent lens, (on the stage of initial cataract on 20 %, on non-mature stage on 26 %, and on mature stage in 2.5 times, p<.01 in all cases). The speed of lens changes progression is directly connected with the degree of vitamin E and C changes. During rapid progression of lens opacifications, in comparison with slow development of cataract, the levels of vitamin E and C are decreased on 9 and 15 % accordingly, >.0 5 in both cases. The results which showed more significant decrease in concentration of vitamin E and C in patients with rapid development of cataract are in accordance with our data about significant decrease of these antioxidants during subcapsular cataracts. This could be an explanation of the very common fact of especially subcapsular cataracts rapid development. The found facts evident the role of antioxidant status in pathogenesis of age-related cataract. Taking into consideration all the known facts about the role of vitamin E in cell defense mechanisms from free radicals processes, and also it's synergism with vitamin C, these data prove the results, which show the structural-functional abnormalities on the level of membrane structures of the cell, during age-related cataract.

Cytotoxic effect of cigarette smoke and its modification by a thiol/selenium antioxidant complex

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Reactive free radicals derived from the burning of cigarettes contribute to tobacco related diseases, including heart and lung disease and cancer. Antioxidants suppress oxidative stress in smokers and help protect cells. Previous studies have shown that thiol antioxidants neutralize oxidants and aldehydes in CS. This study compares cytotoxicity in fetal fibroblasts and in lymphocytes from exposure to CS of control cigarettes and from same brand with an antioxidant complex incorporated in the filter. Methods: A smoking device was used to allow CS to be collected and placed on a confluent cell line of WI-38 fetal fibroblasts and on human lymphocytes. Fibroblasts were monitored via Alamar Blue determining cell viability as ability of functioning mitochondria to reduce the dye. Lymphocyte survival was assessed by the Trypan Blue exclusion test using haemocytometer counting. The test cigarette was prepared with an antioxidant complex in a liposome composed of L-glutathione, N-acetyl-L-cysteine and L-selenomethionine incorporated in the plasticizer application during the manufacture of the filter. Control and test cigarettes had the same tobacco rod. Results: Fibroblast viability from CS exposure averaged 60% and 77% at 24 and 48 hours compared to 95% and 100%, respectively, for the antioxidant treated CS. Lymphocyte survival averaged 65%, 40%, 20% and 8% at 20, 40, 60 and 80 minutes of exposure to the smoke compared to 100%, 82%, 40% and 15% survival of lymphocytes, respectively, for the antioxidant treated CS. Conclusions: An antioxidant complex incorporated in the filter of a cigarette decreases acute cell mortality of fetal fibroblasts and of human lymphocytes after exposure to CS. This antioxidant application in a cigarette filter represents a method to reduce toxicity to cells of inhaled oxidants and aldehydes in cigarette smoke.

Genistein arrests cell cycle and induces apoptosis 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 breast cancer cells support a role for genistein in the modulation of cell cycle leading to inhibition of cell proliferation. We investigated the modulatory effects of genistein, at both physiological and pharmacological concentrations, on cell cycle checkpoints and apoptotic cascade in T47D breast cancer cells. Genistein, at physiologically achievable concentrations (5-10μM): (i) induced p21 (ii) downregulated the antiapoptotic protein, Bcl-xl, and (iii) upregulated mitochondria-associated apoptotic proteins including caspase 9, procaspase 3, and Apaf-1 (apoptotic protease activating factor) and (iv) induced G2-M phase cell cycle arrest after 4 days at 25μM. However, relatively higher concentrations of genistein were required to inhibit proliferation of T47D cells. Similarly, TUNEL staining revealed apoptotic cells in cells treated with 25μM of genistein or greater after 24 hours. The kinetic of these changes was faster with a pharmacological concentration of 100μM of genistein, including cell cycle arrest at 48 hours and p21 induction with a concomitant decrease in cdc-2 activity and protein expression. These data were further supported by a dose- and time- dependent inhibition of cell proliferation. These results suggest that at lower doses genistein is effective in modulating key proteins involved in mitochondria-linked apoptosis. However, genistein causes p21 induction leading to G2-M phase cell cycle arrest and subsequent cell proliferation at 25μM or higher.

Lycopene effects on primary normal human prostate epithelial cells *in vitro*

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Lycopene is believed to protect against development of prostate cancer. So far, experimental studies have focussed on the effects of lycopene on prostate cancer cells. However, it was hypothesized that lycopene may exert important beneficial effects on normal prostate tissue, for example during prostatic hyperplasia and chronic inflammation of the prostate gland, which have both been suggested as neoplastic percursors. Thus, we investigated the effects of lycopene on primary normal prostate epithelial cells in culture. Assays were performed to determine any cytotoxic effects of lycopene treatment, cellular uptake of lycopene and effects on cell proliferation ([3H]-thymidine incorporation). No cytotoxicity of lycopene (≤5 µM) was observed. Lycopene at concentrations of

1 µM and higher strongly inhibited proliferation of primary prostate epithelial cells. We further studied effects of lycopene in an inflammatory model in PrEC cells using LPS and TNF-α as pro-inflammatory stimulators. No induction of a cellular stress response (e.g. heme oxygenase-1) and no effects of lycopene were observed in these cells. From the presented results it was concluded that lycopene at physiologically relevant doses has anti-proliferative effects in primary cultured normal prostate epithelial cells suggesting a possible role in prevention of prostate hyperplasia. Effects of lycopene in inflammatory processes of the prostate need further investigation.

Modulation of matrix metalloproteinase-9 expression in pulmonary epithelial cells by nitric oxide

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Inflammatory lung diseases are associated with increased production of matrix metalloproteinase-9 (MMP-9) from infiltrating granulocytes or from the respiratory epithelium, which produces MMP-9 in response to proinflammatory cytokines such as TNFα. Since inflammatory conditions also result in increased expression of inducible nitric oxide (NO) synthase (iNOS), and NO has been proposed to regulate MMP-9 gene expression and activation, we investigated the involvement of NO or its metabolites on MMP-9 expression in the airway epithelium. Human bronchial epithelial cells were used to investigate the effects of NOS inhibition, iNOS transfection, or exogenous NO-donors on cytokine-induced MMP-9 expression. The results indicated that NOS inhibition or addition of NO-donors did not significantly affect MMP-9 expression, although addition of *S*-nitrosothiols dramatically inhibited cytokine-induced MMP-9 expression, which was related to cellular GSH status and potentiated by depletion of cellular GSH. Cytokine-induced MMP-9 expression involves the activation of NF-κB, and *S*-nitrosothiols, but not spermine NONOate, were found to inhibit the nuclear translocation and DNA binding activity of NF-κB. In summary, cytokine-induced lung epithelial MMP-9 expression is regulated by *S*-nitrosothiols, illustrating an additional mechanism by which nitrosative stress may affect epithelial injury and repair processes during conditions of airway inflammation.

Redox Regulation of α-Tubulin Tyrosination by Nox1

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Whereas Nox1, a novel homolog of the phagocyte NADPH oxidase (gp91phox) confers a proliferative and transformed phenotype to cells of non-phagocytic origin, the molecular mechanisms underlying these effects are not well characterized. Microtubules are important for many aspects of mammalian cell biology including growth, migration and signaling, and α-tubulin is unique in that it undergoes a reversible posttranslational modification whereby the C-terminal tyrosine residue is removed and readded. We have hypothesized that oxidants produced by Nox1 may induce proliferation by modulating tubulin post-translational modifications. Consistent with this hypothesis, 3T3 cells transfected with Nox1 expressed higher levels of detyrosinated and acetylated α-tubulin compared to wild-type cells and the extent of α-tubulin detyrosination paralleled the degree of proliferation. This effect was reversed by catalase co-transfection. In addition, human prostate cancer cell line (LNCaP) not only shared morphological characteristics with Nox1 transfected cells, but also expressed Nox1 and exhibited high level of detyrosinated and acetylated tubulin. It was also found that 3-nitrotyrosine, which is irreversibly incorporated into the C-terminus of α-tubulin, blocked the proliferative response of Nox1 transfected cells, suggesting that α-tubulin detyrosination state may be an important factor in the proliferative response. These results suggest that Nox1 can regulate α-tubulin tyrosination state, which may play an important role in cellular proliferative diseases such as cancer.

Leukoisoprenochrome-*o*-semiquinone formation in freshly isolated adult rat cardiomyocytes

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Sustained high levels of circulating catecholamines may induce cardiotoxicity. There is increasing evidence that this could result from catecholamine oxidation into *o*-quinones and aminochromes, which is catalysed by transition metals. In fact, it has already been shown that Cu^{2+}-induced oxidation of the β-agonist isoproterenol (ISO) in isolated cardiomyocytes: i) decreases cell viability, ii) induces intracellular glutathione depletion by glutathione oxidation and adduct formation and iii) reduces selenium dependent glutathione peroxidase, glutathione reductase and glutathione-S-transferase activities (1,2). It was also observed a decrease of ISO and an increase of isoprenochrome levels in both, extracellular medium and cells (2). However, there is still scarce information concerning the target in the cardiomyocytes where the ISO oxidation occurs and induces toxicity. The aim of this work was to study and localize ISO oxidation in cardiomyocytes.

Freshly isolated rat cardiomyocytes were incubated with 10 mM ISO in the presence of 20 μM Cu^{2+} for 4 hours. A control sample without ISO was also performed. A time-dependent decrease of cardiomyocytes viability evaluated by trypan blue exclusion was observed in ISO treated samples. After 3 and 4 hours of incubation, samples of cell suspension were centrifuged and the pellet (cells) was washed three times with freshly incubation solution. Samples of first supernatant (extracellular medi-

um), last wash solution and cells were analysed by ESR. All samples were supplemented with Mg^{2+} (5 mM, final concentration) before ESR analysis.

An ESR signal, assigned to leukoisoprenochrome-*o*-semiquinone (LI-*o*-semiquinone), was found in both, extracellular medium and cells after 3 and 4 hours of incubation. The levels of LI-*o*-semiquinone were higher after the 4 hours of incubation period but reduced by adding 5 mM of GSH. LI-*o*-semiquinone was not found either in control samples or in any last wash solutions. Furthermore, by adding 20 mM potassium chromium(III) oxalate the signal of LI-*o*-semiquinone was broadened in the extracellular medium with no significant changes in the cells. These results strongly suggest the formation of LI-*o*-semiquinone in both, the extracellular medium and cells. In order to know where the cellular LI-*o*-semiquinone is located, the cells were disrupted and centrifuged. The LI-*o*-semiquinone was found mainly in the pellet, which indicates its presence in cellular membranes.

In conclusion, the present study seems to indicate that ISO oxidation occurs in the cellular membranes, which explain the observed and previously described toxicity of Cu^{2+}-induced ISO oxidation in isolated cardiomyocytes.

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Antioxidant Action of 1-(11-Selenadodecyl)-glycerol and 1-(11-Selenadodecyl)-3-(6-hydroxy-2, 5, 7, 8-tetramethyl-chromane-2-acyl)-glycerol against lipid peroxidation

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We studied *in vitro* the inhibitory effect on lipid peroxidation of two newly synthesized selenium compounds 1-(11-selenadodecyl)-glycerol (SeG) and 1-(11-selenadodecyl)-3-(6-hydroxy-2,5,7,8-tetramethylchromane-2-acyl)-glycerol (SeTrG), the latter containing the antioxidants selenium and trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid). SeTrG showed peroxyl radical scavenging activity. SeG reduced methyl linoleate hydroperoxides in methanol solution and in Fe2+/AA catalyzed methyl linoleate micelles oxidation. SeTrG was more effective than SeG against Fe2+/AA catalyzed methyl linoleate micelles oxidation. In biological environment like rat plasma the selenium compounds suppressed the formation of conjugated diene hydroperoxides in the AAPH-induced oxidation. In MLV suspensions SeG and SeTrG spared α-TOH in AAPH and AMVN induced oxidations; SeTrG was effective sparing α-TOH in SIN-1 oxidation and SeG didn't have effect. In rat plasma SeTrG spared α-TOH consumption in the AAPH-, MeO-AMVN- and SIN-1-induced oxidations and SeG didn't. The inhibitory effects of SeG and SeTrG could be explained with the particularities in their structures, the different microenvironment, and their efficiency against the oxidants.

A thiol/selenium antioxidant complex in a cigarette filter ameliorates gas phase smoke's modification of salivary proteins

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Cigarette smoke (CS), which is injurious to salivary proteins, is associated with various oral pathologies and cancer. Previous studies have shown that saliva exposed to CS increases protein carbonyls from oxidants and aldehydes reacting with salivary proteins. Thiol antioxidants reduce CS induced carbonyls. This study evaluated the effects of a thiol antioxidant complex placed in the filter of a cigarette on salivary protein carbonyl levels. Methods: Saliva was collected from non-smokers and exposed to CS from research (IR4F, University of Kentucky), conventional and same brand cigarettes, the latter with an antioxidant complex incorporated during the manufacture of the filter. The antioxidant complex in a liposome was composed of L-glutathione, N-acetyl-L-cysteine and L-selenomethionine. Protein carbonyls were assessed by standard biochemical technique measured in nmoles/mg protein, by Western blot analysis using anti-DNPH antibodies and by Thermochemiluminescence (TCL Lumitest, Ltd, Haifa, Israel) which measures photon signals from oxidated “excited” carbonyl species. Three different time points were taken at 300 seconds from TCL measurements. Results: Production of protein carbonyls after CS exposure to the research and conventional cigarettes were significantly higher than from antioxidant treated CS. TCL showed lower levels of carbonyls from the antioxidant treated CS at the two-hour post exposure period compared to those elicited from the control cigarettes at the three different time points (50, 100, 150 seconds) of TCL excitation. Conclusions: An antioxidant complex in the filter of a cigarette neutralizes oxidants and volatile aldehydes that damage salivary proteins as evidenced by a reduction in the production of protein carbonyls. This antioxidant application in the filter of a cigarette may render inhaled cigarette smoke to be less toxic to smokers.

Co-operation between antioxidants in protection against photo-oxidative damage

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The outer retina – photoreceptors and retinal pigment epithelium (RPE) – is inherently at risk of photo-oxidative damage due to high oxygen tension, substantial fluxes of visible radiation and the presence of potent photosensitisers. The aim of this study was to determine the efficiency of individual antioxidants: zeaxanthin (ZEA), α-tocopherol (TOH), and ascorbate (AH), and combinations thereof, in protecting RPE cells against photo-oxidative damage. To model the *in vivo* situation, where RPE cells are adjacent to photoreceptor outer segments, photo-oxidative damage was induced by 60 minutes irradiation of ARPE-19 cells in culture with blue light in the presence of liposomes containing retinal. The effect of antioxidants on plasma membrane permeability and cell viability was tested by propidium iodite staining and MTT assay, respectively. Exposure of RPE cells to blue light and 0.5 mM retinal led to decrease of cell viability to 20%. Single antioxidants offered only limited protection. Increasing concentrations of lipophilic antioxidants in liposomes, in the range of 0.5-4 μM for ZEA and 10-40 μM for TOH, did not result in a substantial increase in protection. Increasing the concentration of AH above 0.5 mM led to prooxidant effects. Cells enriched in both ZEA and TOH were protected in a synergistic way when compared with cells enriched with single antioxidants. Synergististic effects were also observed for combinations of TOH and ZEA in liposomes. The greatest protection was offered by combinations of AH with ZEA in liposomes, while combinations of TOH/AH were the least effective. In conclusion, combinations of a singlet oxygen quencher with a free radical scavenger offer better protection against photo-oxidative damage than increasing concentrations of single antioxidants.ß

iNOS-induction by LPS is attenuated in α–tocopherol transfer protein knockout mice

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α–Tocopherol (α–T), a potent, lipid-soluble antioxidant, may be critical for protection against oxidative injury. In the lungs, inadequate antioxidants not only increases susceptibility to oxidant damage, but the oxidant injury may induce an inflammatory response that will result in further oxidant production by infiltrating neutrophils. Mice with a deletion in the α−tocopherol transfer protein gene (*Ttpa-/-*) have lung α−tocopherol concentrations 1/10 of wildtype control mice (*Ttpa+/+*) and therefore were used to study the role of α-tocopherol in lung inflammation. Mice were injected intraperitoneally with LPS (12 h, 10 mg/kg). Animals were sacrificed; the lungs lavaged for determination of inflammatory cells; plasma, lung and liver tissues were obtained for analysis. Lung inflammation was confirmed in lung lavage fluid by influx of neutrophils and/or MIP-2 (neutrophil chemoattractant) analysis and was found to be elevated in LPS mice, but not significantly different between *Ttpa-/-* and wildtype mice. Inducible NOS (iNOS), an indicator of inflammation, was induced by LPS in both groups as determined by quantitative western blots, but α-T deficiency attenuated this expression. Subsequently released nitric oxide (measured as NO_2+NO_3) was found to be increased by LPS injection in lung lavage and plasma, but to a lesser extend in *Ttpa-/-* mice. Heme-oxygenase-1 (HO-1) is induced by oxidative stimuli and may protect against oxidative stress. *Ttpa-/-* mice showed a trend towards increased HO-1 expression (measured by western blots) in the lung. After injection of LPS, lung HO-1 protein was found to be increased in both groups of mice, but a trend towards reduced HO-1 was observed in *Ttpa-/-* compared to wildtype mice. Additionally, isolated neutrophils from bone marrow of *Ttpa-/-*

mice showed increased PKC dependent respiratory burst when stimulated with PMA (100 ng/ml) compared to wildtype animals. Our results suggest that α–T deficiency may impair induction of inflammatory genes and mediators possibly through enhanced PKC activation. Since induction of NOS or HO-1 are thought to protect cells from further stress, these findings indicate that α–T may play a role in lung defense against inflammation independent of its anti-oxidant properties.

Impact of carotenoid beadlets on stability and uptake of carotenoids in cell cultures

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Protective effects of carotenoids against serious disorders such as cancer, heart disease and degenerative eye disease have been recognized, and have stimulated research into their role as antioxidants and as regulators of the immune system response. Cell culture systems provide an opportunity to evaluate effects of carotenoids on molecular and cellular processes. Since carotenoids are not water-soluble, the delivery method in cell culture presents unique challenges. Different methods can affect the miscibility, availability, and sensitivity of carotenoids to degradation. The objective of this study was to determine how water-dispersible preparations of carotenoid containing beadlets can affect the stability and facilitate cellular uptake. In this regard, suitable in vitro model systems were established, necessary analytical methods were developed, stability and availability of the lycopene, beta-carotene, lutein or astaxanthin carotenoid beadlets during cell culture incubations were determined and the cell-specific uptake of carotenoids for human peripheral blood mononuclear cells, human umbilical vein endothelial cells, and monocytes were established. For the first time we show that extraction is not necessary if analyzing the water-dispersible carotenoids in medium and they can be also treated like water-soluble substances and injected into HPLC directly. The results show that the beadlets offer a reasonable compromise in terms of stability and uptake efficiency; when incubated in medium without light for 24 h, the extent of uptake of the four carotenoids at a concentration of 1 μM was similar in each cell culture system. This is at variance with the reports showing large differences in uptake of different carotenoids delivered by the THF method.

Ubiquitin-independent degradation of oxidized proteins by the proteasome

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Aging and a number of age-related pathologies are accompanied by the accumulation of oxidatively modified forms of proteins. Mammalian cells exhibit limited direct repair mechanisms and oxidatively damaged proteins appear to undergo selective proteolysis, primarily by the proteasome. 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*. The primary aim of this study is to determine if the degradation of oxidized proteins by the proteasome *in vivo* is dependent on ubiquitin conjugation. 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 temperature sensitive conditional mutation for the ubiquitin-activating enzyme E1, which controls the first step in the ubiquitin conjugation pathway. 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 selective proteasome inhibitors. In separate, *in vitro* assays for ubiqutin conjugation of native and oxidized substrates, we have seen that progressive oxidation does not promote more ubiquitinylation. These data suggest that the degradation of oxidized proteins in cells is ubiquitin-independent and may be conducted by the 20S proteasome.

Sptrx-2 and Txl-2 are fusion proteins composed of thioredoxin and NDP-kinase domains prominently expressed in mammalian testis

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Thioredoxins are a growing family of proteins that function as general protein disulfide reductases. During the recent years, our group has identified several novel members of this family prominently expressed in mammalian testis and sperm. Among these, Sptrx-2 and Txl-2 have in common their domain organization composed of one thioredoxin domain followed by three or one NDP-kinase domain, respectively. Sptrx-2 mRNA is exclusively expressed in mammalian spermatocytes while Txl-2 mRNA is predominantly found in testis and lung, although at very low levels. Recombinant Sptrx-2 and Txl-2 proteins display neither thioredoxin nor kinase activity *in vitro*. Immunohistochemistry in testis sections identifies Sptrx-2 protein in late spermatocytes and spermatids and immunogold electron microscopy localizes Sptrx-2 protein in the sperm fibrous sheath. Sptrx-2 expression pattern differs from that of Sptrx-1 in the fact that Sptrx-2 remains as a structural component of the fibrous sheath in ejaculated sperm. In turn, Txl-2 protein is found in the spermatid manchette (a microtubular structure associated to the nucleus of elongating spermatids) and in the cilia of the airway epithelium in lung. Preliminary data indicate that Txl–2 binds microtubules *in vitro*. The association of thioredoxins and NDP kinase proteins in spermatozoa suggests an important advantage for mammalian internal fertilization. In addition, the presence of Txl-2 in lung cilia indicates a possible modulatory function of the cilia beating which may be important in some pathological situations such as the so-called “immotile cilia syndrome”.

Effect of FPP on the resistance of rats against infection with Salmonella

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Studies performed in other laboratories suggest that FPP (Fermented Papaya Preparation ; made by OSATO Int. Inc. Japan) stimulates the *in vitro* NO response of macrophages. NO is an important factor in the defense against pathogens, including salmonella. In studying the effect of FPP on the resistance of rats against infection with salmonella, the tests were done upon oral administration of salmonella. After two weeks of the examination, the severity of the infection was followed by the registration of salmonella excretion in feces as a marker for colonization resistance, and NO_2 and NO_3 (Nox) excretion in urine as a sensitive and quantitative marker for bacterial translocation to extra-intestinal organs. The fecal excretion of salmonella was a little change between the FPP group and control at day 7 which was unexpected. During the infection, the NOx excretion increased as expected. Although the NOx excretion was on average higher, the effect was only significantly higher at day 4 of the infection. At day 7 of the infection, the mean relative liver weight of the FPP group was significantly lower. This indicative for improved liver functioning, but needs further investigation.

Ascorbate reduces ischemia and reperfusion injury

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In a series of experiments we have provided *in vivo* evidence of the salvage effect of 32 mM of ascorbate on postischemic reperfusion in rat skeletal muscle, gastric mucosa and heart (1, 2, 3). Treatment with ascorbate during postischemic reperfusion enhanced cardiolipin levels which correlated well with improved ATP synthesis as evidenced by *invivo/invitro* MRS (4). Furthermore, a synthesized indeno-indole compound (H290/51, Astra Hässle, SE) that acts as a lipid peroxidation chain reaction terminator was studied. Like vitamin E, this compound can recycle with ascorbate but in a more powerful way. Treatment with H290/51 improved the postischemic recovery of the rat heart, and of the rabbit kidney in a similar manner to ascorbate yhat is, with regard to functional and energetic parameters (3, 5). In a study on the ischemic and reperfused rabbit kidney, a combination of H290/51 and ascorbate was tested. Four groups were investigated (1.Control, 2. Treated with H290/51, 3. Treated with ascorbate, 4. Treated with ascorbate (1g/kg) + H290/51 (20mmol/kg)) Experiments were carried out *in vivo* by MRS/MRI at 2.35 Tesla continuously. Initially, a ^{1}H MR image of the kidney was aquired and subsequent 31P MRS spectra were recorded selectively from a 2.3 ml volume comprising the cortex and the outer medulla. Concentrations of ATP, AMP, inorganic phosphate, phosphomonoesters/diesters glucose phosphates were calculated.The group treated with both ascorbate and H290/51 had the best recovery during postischemic reperfusion. The ATP/Pi ratios were significantly higher in group 4 compared to all other groups ($p<0.05$, $n=8$).Conclusion: Both the vitamin E analogue and ascorbate could reduce cellular damage during postischemic reperfusion. However, a combination of the two compounds had the most powerful antioxidant effect. Probably by a mechanism protecting unsaturated fatty acids and particularly cardiolipin from oxidation

by regenerating vitamin E at the surface of the inner mitochondrial membrane. This might explain the observed improvement in mitochondrial function, PCr, ATP and intracellular pH levels.

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Ozone exposure activates oxidative stress responses in murine skin

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Ozone (O_3) is among the most reactive environmental oxidant to which skin is exposed. O_3 exposure has previously been shown to induce antioxidant depletion as well as lipid and protein oxidation in the outermost skin layer, the stratum corneum, but little is known regarding the potential effects of O_3 on the skin epidermis and dermis. To evaluate such skin responses to O_3, SKH-1 hairless mice were exposed for 2 h to 8.0 ppm O_3 , 6 h to 0.8 ppm or to ambient air. O_3 exposure caused a significant increase in 4-hydroxy nonenal-protein adducts, compared to the skin of air exposed control animals. Furthermore, O_3 caused a rapid up-regulation of HSP27 and HSP70 after 8.0 ppm of O_3, and a more delayed induction of HSPs after 0.8 ppm of O_3. In addition, we observed increased level of iNOS and MMP9, suggesting an induction of inflammation. It is concluded that skin exposure to O_3 not only affects antioxidant levels and oxidation markers in the stratum corneum, but also induces oxidative stress responses in the active cellular layers of the skin, most likely by indirect mechanisms, since O_3 itself is to reactive to penetrate far into the tissue. Thus, skin exposure to O_3 could potentially impact or cellular processes involved in skin, development, aging, wound healing or cancer development.

Effect of SDS micelles on the acid-base and hydration kinetics of malvidin 3-glucoside (Oenin)

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Anthocyanins are natural water-soluble pigments responsible for the color of many fruits and flower petals. These compounds are potentially interesting antioxidant agents against cancer and cardiac diseases.[1] However, their chemical and photochemical properties are poorly understood. Oenin is a representative, naturally occurring anthocyanin that is found in European casts of red grapes.[2] In this work,[3] the causes for the huge stabilization of color of the natural anthocyanin Oenin in SDS micelles were investigated. The four rate constants involved in the acid-base and hydration reactions of Oenin in SDS were measured using light-jump and pH-jump techniques. It was found that the c.a. 3 orders of magnitude decrease of the equilibrium constants of the acid-base and the hydration reactions from water to SDS micelles is responsible for color stabilization (present at neutral pH). The decrease of the equilibrium constants is only partially due to the increase of the back-reaction rates of the acid-base and hydration processes, resulting from the higher proton concentration at the SDS micelle surface relative to water. Interestingly, the major contribution to the decrease of the acid-base equilibrium constant is the strong decrease of the deprotonation (kd = 2 × 10^5 s^{-1}) rate constant in SDS micelles with respect to water (kd = 5.0 × 10^6 s^{-1}). Also the hydration (kh = 4.6 × 10^{-3} s^{-1}) rate constant in SDS micelles de-

creases with respect to water (kh = 8.5 × 10^{-2} s^{-1}) but in this case the major contribution to the shift in the hydration equilibrium comes from the back reaction (k-h = 34 $M^{-1}s^{-1}$ in water against k-h = 7.1 x 10^3 $M^{-1}s^{-1}$ in SDS). This result is consistent with the expected stabilization of the positively charged flavylium cation by the negative charge of the SDS micelle, with respect to both the anhydrobase and the hemiacetal forms of Oenin.

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Ingestion of fermented beverages increases plasma antioxidant activity

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Background: Moderate consumption of alcoholic beverages, or antioxidants may reduce risk of conditions which incorporate an oxidative process in their pathogenesis, reducing risk of several diseases associated with ageing, including cataract and atherosclerosis. Objectives: To test whether alcoholic beverages have antioxidant properties and whether their consumption can affect the antioxidant activity of plasma. Design, interventions, and main outcome measures: Red wine, lager beer, stout (alcoholic and alcohol-free), and a solution of alcohol were compared for (1) their antioxidant activity in vitro (2) their polyphenol content, (3) the ability of stout to affect the electron spin resonance spin (ESR) spectra of reactive oxygen species, (4) the plasma antioxidant activity in human volunteers following consumption. In addition, preliminary investigations of lag times for copper-catalysed LDL + VLDL oxidation were conducted on plasma samples after stout consumption. Results: Red wine, lager beer, stout(5% alcohol v/v, and alcohol-free) were effective antioxidants in vitro. Stout strongly suppressed the spin-trapped superoxide signal, and reduced the hydroxyl radical signal. After ingestion, all beverages except the solution of alcohol increased significantly the antioxidant activity in plasma samples. Averaged over the 240 minute period, all beverages except the alcohol solution showed similar plasma antioxidant activities. 30 minutes after ingestion of stout a significant increase in lag times for copper-catalysed LDL + VLDL oxidation was found. Conclusions: Antioxidant activity of beverages may be related to health benefits. Support: Guinness, Labatt Brewing

Oxidant Stress in iron-overloaded subjects with Thalassemia or sickle cell disease

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Biomarkers for oxidant stress metabolism were measured to test the hypothesis that differences in iron metabolism and oxidant damage exist between _-thalassemia (THL) and sickle cell disease (SCD). Plasma levels of malondialdehyde (MDA), a marker of lipid peroxidation, were determined by GC-MS. Assays detecting oxidative damage to DNA of white blood cells are in progress. Plasma tocopherols were measured using HPLC with electrochemical detection. Liver biopsies were also taken and analyzed by transmission electron microscopy (TEM), for the detection of iron and morphological changes. We have found elevated levels of MDA and γ-tocopherol in the plasma of both THL and SCD patients that positively correlate with serum ferritin. Examination of the TEM micrographs from the liver biopsies revealed elevated iron deposits in the livers of the SCD and THL groups relative to normal liver, which is consistent with the iron overload disea e process. Thus, there is elevation of MDA in THL and SCD that is dependent on the rise in serum ferritin, suggesting iron induced oxidant stress in these iron-overloaded patients. (Supported by NIH grant R01-DK5777-01)

Oxidation of β-carotene induces formation of 8-hydroxy-deoxyguanosine in calf thymus DNA by the production of reactive oxygen species

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Oxidized β-carotene (OBC) has been shown to have pro-oxidant and pro-carcinogenic effects. We recently reported that addition of OBC (obtained by dissolving BC in tetrahydrofuran and incubated at 60¢J for 1h or at 37¢J for 9h in air), but not of BC, to calf thymus DNA or human foreskin fibroblasts induces DNA damage measured as formation of 8-hydroxy-deoxyguanosine (8-OH-dG). However, the mechanism by which OBC induces the formation of 8-OH-dG is unclear. To examine our hypothesis that reactive oxygen species (ROS) are involved in OBC-induced formation of 8-OH-dG, we used indirect assays of ROS by adding iron chelators or radical scavengers during incubation (37¢J for 3h) of calf thymus DNA with OBC. The results show that addition of desferal, a strong iron chelator, at 50mM decreased the level of 8-OH-dG by 80%, indicating the involvement of adventitious iron ions. Peroxyl radical and alkoxyl radical also played a major role because butylated hydroxytoluene (a peroxyl radical scavenger) and diphenylamine (an alkoxyl radical scavenger) strongly decreased 8-OH-dG levels. Singlet oxygen ($^1O_2$) was also involved because NaN_3 (a scavenger of both •OH and $^1O_2$) but not DMSO (a •OH scavenger) significantly inhibited 8-OH-dG formation. Superoxide anion appeared to play a minor role, if any, because SOD only slightly, but not significantly, inhibited 8-OH-dG formation. In the absence of calf thymus DNA, autoxidation of BC at either 60¢J for 1h or 37¢J for 9h produced aldehydes (measured as cyclohexane-dione-reactive substances) which were also markedly inhibited by iron chelators and radical scavengers. In addition, MDA was pro-

duced by autoxidation of BC, suggesting chain-propagated reactions during autoxidation. Thus, the present study demonstrates that ROS produced by BC autoxidation, possibly by metal ion-catalyzed breakdown of pre-formed BC peroxides (BCOOH), induce the formation of 8-OH-dG in calf thymus DNA. The presence of BCOOH is suggested by the formation of MDA, which did not induce 8-OH-dG formation, but can interfere with the MDA assay as a marker of lipid peroxidation.

Key words: Oxidized β-carotene, DNA damage, 8-OH-dG, reactive oxygen species (ROS), transition metal ion.

Pyrrolidine dithiocarbamate is a potent antioxidant against hypochlorous acid-induced protein damage

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The dithiol compound pyrrolidine dithiocarbamate (PDTC) is a putative antioxidant that has been used frequently in biological research. However, its antioxidant properties are not well characterized. In this study, we investigated in detail the antioxidant potential of PDTC against protein damage induced by hypochlorous acid (HOCl), a potent chlorinated oxidant generated by activated phagocytes. The effects of PDTC were compared to those of GSH and *N*-acetylcysteine.

PDTC markedly and in a concentration-dependent manner inhibited HOCl-induced inactivation of α1-antiproteinase, protein carbonyl formation in bovine serum albumin, and oxidation of human low-density lipoprotein. In each of these three assay systems, PDTC was two to three times more potent than GSH and *N*-acetyl cysteine, while diethyl dithiocarbamate (DDTC) was about as effective as PDTC. For example, the concentrations required for 50% inhibition of HOCl-induced inactivation of α1-antiproteinase were 13 μM PDTC and 38 μM GSH, and for inhibition of HOCl-induced protein carbonyl formation 70 μM PDTC and 160 μM GSH. Furthermore, based on two quantitative methods, *i.e.*, oxidation of ferrocyanide and chlorination of monochlorodimedon, one molecule of PDTC was able to scavenge up to eleven molecules of HOCl, whereas one molecule of GSH only scavenged four molecules of HOCl

These data demonstrate that PDTC and DDTC are potent antioxidants against HOCl-induced protein oxidative damage, suggesting that these and other dithiocarbamates might be useful in the prevention and treatment of inflammatory conditions. (Supported by NIH grants ES11497 and 00210)

Metal-independent hydroxyl radical production by carcinogenic pentachlorophenol metabolites and hydrogen peroxide: What is the mechanism?

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Tetrachloro-1,4-benzoquinone (TCBQ) has been identified as a major genotoxic metabolite of the widely used wood preservative pentachlorophenol (PCP). PCP is now considered to be ubiquitously present in the environment and even found in people who are not occupationally exposed to it. PCP has been listed as a priority pollutant by the U.S. EPA, and classified as a group 2B environmental carcinogen by the International Association of Research on Cancer (IARC). Recently, we found that hydroxyl radicals ($\cdot$OH) are produced by TCBQ and hydrogen peroxide (H_2O_2) independent of transition metal ions. The production of $\cdot$OH was measured by secondary radical ESR spin-trapping techniques, where $\cdot$OH form methyl radicals upon reaction with dimethyl sulfoxide, and the methyl radicals are subsequently detected by ESR spectroscopy as the adduct with the spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO). In contrast, no $\cdot$OH was detected from H_2O_2 and tetrachlorohydroquinone (TCHQ), the reduced form of TCBQ. However, if TCHQ was quickly oxidized to TCBQ by addition of myeloperoxidase, $\cdot$OH could be detected. The antioxidants, ascorbate, dihydrolipoic acid and glutathione, completely inhibited the production of $\cdot$OH by H_2O_2 and TCBQ. No correlation was found between $\cdot$OH and tetrachlorosemiquinone anion radical (TCSQ-$\cdot$) formation. Thus, the production of $\cdot$OH by TCBQ and H_2O_2 may not be through a previously proposed metal-independent organic Fenton reaction, TCSQ-$\cdot$ +

H_2O_2 —> TCBQ + · OH + OH-, in which TCSQ-· substitutes for ferrous iron in the classic, metal-dependent Fenton reaction. Based on our recent results and other literature reports, a new mechanism is proposed: H_2O_2 may react with TCBQ or TCSQ-· by a nucleophilic reaction, forming a phenylhydroperoxide intermediate. Because the oxygen-oxygen bond of phenylhydroperoxide is weak due to the stability of the resulting phenoxyl radical, · OH may be produced by homolytic fission of the hydroperoxide group. Further studies are needed to investigate this hypothesis.

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α-Lipoic acid decreases the free thiol content of the insulin receptor and protein tyrosine phosphatase 1B in 3T3-L1 adipocytes

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The role of intracellular redox status and the thiol content of the insulin receptor during glucose uptake into 3T3-L1 adipocytes were investigated. Insulin- or α-lipoic acid treatment of the cells stimulated glucose uptake, increased tyrosine phosphorylation of the insulin receptor, and decreased free thiol content of its β-subunit. Insulin- or α-lipoic acid-stimulated glucose uptake was inhibited i) by alkylation of intracellular, but not extracellular, thiol groups down stream to the insulin receptor activation, and ii) by NADPH oxidase inhibitors at the level of the insulin receptor autophosphorylation. α-lipoic acid treatment also inhibited total cellular protein tyrosine phosphatase (PTP) activity and decreased the free thiol content of PTP 1B, which is implicated in negative regulation of insulin signaling cascade. These findings support the importance of intracellular redox status in glucose transport and indicate that thiol groups present in insulin receptor β-subunit and PTP 1B are targets of α-lipoic acid action. The common mechanism whereby insulin or α-lipoic acid stimulates glucose uptake could be by H_2O_2 production and its effect on insulin signaling pathway.

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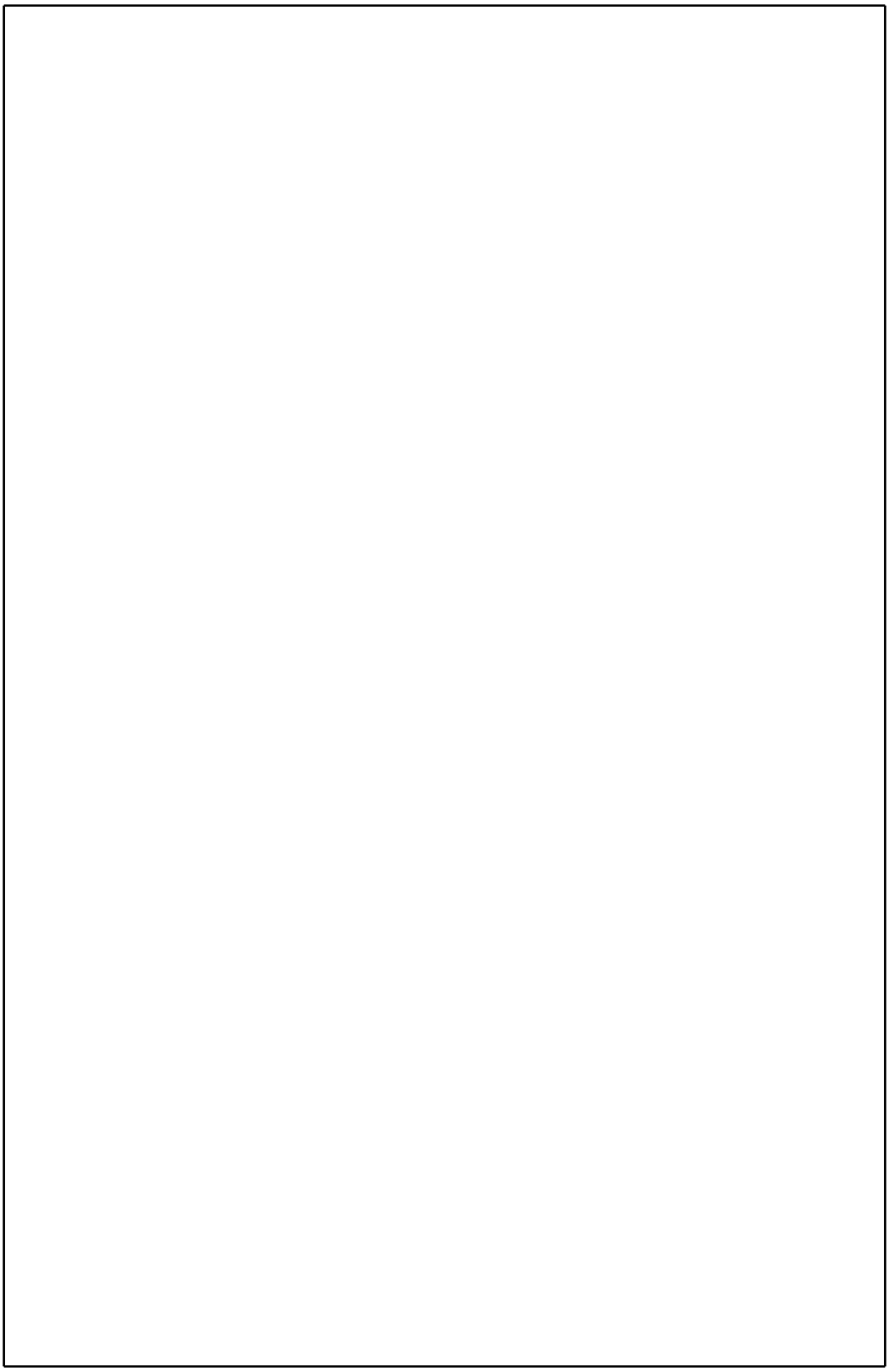

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