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OXIDANTS AND ANTIOXIDANTS IN BIOLOGY

BOOK OF ABSTRACTS

FEBRUARY 6–9, 2003

HOTEL MONASTERIO DE SAN MIGUEL

EL PUERTO DE SANTA MARÍA, CÁDIZ, SPAIN

OXIDANTS AND ANTIOXIDANTS IN BIOLOGY

A MEETING IN HONOR OF LESTER PACKER

FEBRUARY 6–9, 2003
EL PUERTO DE SANTA MARIA, CÁDIZ, SPAIN

A JOINT MEETING OF
UNIVERSIDAD DE CÁDIZ
GRUPO ESPAÑOL DE RADICALES LIBRES
OXYGEN CLUB OF CALIFORNIA

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KEYNOTE ADDRESS

The antioxidant network: From scavenging free radicals to regulation of genes

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Our research on vitamin E and mechanisms of antioxidant action started in the1960's at the University of California Berkeley, soon introducing an ESR approach that addressed dynamic aspects of chromanoxyl radical reactions within the vitamin E cycle and established a dynamic interplay among key antioxidants. The players were vitamin C, vitamin E, coenzyme Q, glutathione, and the newly discovered "metabolic antioxidant" α-lipoic acid. These *in vitro* studies as well as evidence from cell culture and *ex vivo* models led to the concept of the *antioxidant network*, in which antioxidants interact with- and spare one another. The antioxidant network helps maintain the delicate balance between oxidants and antioxidants, a critical point with implications for health and disease.

The identification of oxidant-sensitive pathways of cell signaling mandated that antioxidants regulate gene expression by different mechanisms: First, by affecting the cell redox status, largely controlled by network antioxidants. Second, specific effects of antioxidants on key targets of the transciptome have been identified. Azzi's report in 1991 of PKC inhibition by α-tocopherol, but not by other vitamin E steroisomers, was a key finding. In the 1990's, we turned our attention toward antioxidant regulation of oxidant-sensitive transcription factors, such as NFκB dependent genes, cell adhesion molecules, and iNOS gene expression. Our findings identified tissue-specific targets of gene regulation by bioflavanoid-rich botanical extracts, such as Gingko biloba EGb 761 and the pine bark extract Pycnogenol. Interestingly, these extracts exhibited greater activity than any of their individual bioflavanoid components, thus suggesting synergistic effects. Global gene expression analysis by high-density oligonucleotide arrays are being used for studies of gene regulation in cell culture and animal tissues treated with EGb 761. Vitamin E- dependent genes in liver and brain have been investigatedin α-tocopherol-transfer protein (TTP) knock out

mice. Cell uptake by carotenoids, cell cycle regulation, and gene expression are being investigated in human prostate cells in order to understand the inhibitory effects of lycopene on cell proliferation. Our findings reveal remarkable tissue specific effects of antioxidants, which apart from their participation in the entire system of antioxidant defense, could not have been predicted *a priori* from knowledge of their *in vitro* reaction mechanisms. Antioxidant- and micronutrient research will increasingly rely on genomic and proteonomic studies to elucidate the molecular basis of their health effects.

SESSION I

REDOX REGULATION OF CELL SIGNALING

Estrogen regulation of mitochondrial function: A unified concept of estrogen-induced neuroprotection and implications for neurodegenerative disease

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We sought to determine the mechanism by which estrogen can promote intracellular Ca^{++} homeostasis and survival in the presence of toxic insults that lead to neuronal death via dysregulation of Ca^{++} homeostasis. Because of the Ca^{++} buffering capacity of mitochondria, we investigated the role of mitochondria in E_2-induced regulation of $[Ca^{++}]_i$. Since Bcl-2 plays a key role in mitochondrial Ca^{++} regulation (15, 16), the effect of E_2 on Bcl-2 expression was also investigated. Results of this investigation, demonstrate that 17β-estradiol (E_2) treatment of hippocampal neurons attenuated the excitotoxic glutamate-induced rise in bulk free $[Ca^{++}]_i$ despite potentiating the influx of Ca^{++} induced by glutamate. E_2-induced attenuation of bulk free $[Ca^{++}]_i$ is dependent upon mitochondrial sequestration of Ca^{++}, which is blocked in the presence of the combination of rotenone and oligomycin, or in the presence of antimycin, which collapse the mitochondrial membrane potential, therefore preventing mitochondrial Ca^{++} transport. Release of mitochondrial Ca^{++} by FCCP following excitotoxic glutamate treatment resulted in a greater $[Ca^{++}]_i$ in E_2-treated cells, indicating an E_2-induced increase in the mitochondrial calcium ($[Ca^{++}]_m$) load. The increased $[Ca^{++}]_m$ load was accompanied by increased expression of Bcl-2, which can promote mitochondrial Ca^{++} load tolerance. These findings provide a novel mechanism of E2-induced neuronal survival by attenuation of excitotoxic glutamate $[Ca^{++}]_i$ rise via increased mitochondrial sequestration of cytosolic Ca^{++} coupled with an increase in Bcl-2 expression to sustain mitochondrial Ca^{++} load tolerance and function. Results of these analyses support the hypothesis that E_2-induced neuroprotection is mediated by attenuation of glutamate-induced $[Ca^{++}]_i$ rise via increased mitochondrial sequestration of Ca^{++} coupled with increased

Bcl-2 expression to promote mitochondrial tolerance of an increased $[Ca^{++}]_m$ load. E_2-induced sequestration of Ca^{++} by mitochondria provides a potential unifying mechanism to explain the neuroprotective effect of estrogens against a broad spectrum of neurotoxic insults. Moreover, increasing mitochondrial calcium sequestration and calcium load tolerability could effectively inhibit activation of the apoptotic cascade thereby increasing neuronal survival and viability. The significance of these findings for estrogen-inducible prevention of neurodegenerative diseases such as Alzheimer's and Parkinson's will be discussed.

Quinone-induced signaling: From intra- to intercellular communication

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Exposure of WB-F344 rat liver epithelial cells to the arylating and redox-cycling quinone menadione (2-methyl-1,4-naphthoquinone, vitamin K_3), led to an up to 75 % decrease in gap junctional intercellular communication (GJC), which was not due to internalization of gap junctions. Rather, the decrease in GJC was found to be due to phosphorylation of connexin 43, which was mediated by activation of the EGF receptor − MEK1/2 − ERK1/2 cascade. Activation of ERK1/2 was demonstrated to be independent of NAD(P)H:quinone oxidoreductase using the inhibitor dicumarol, thus excluding redox-cycling as the major mechanism causing these menadione effects. Both the tyrosine phosphorylation of the EGFR and the inactivation of a tyrosine phosphatase regulating the EGFR were observed, consistent with arylation by menadione being responsible for the signaling events induced. ERK activation was attenuated employing specific inhibitors of the EGFR tyrosine kinase. Similarly, these inhibitors as well as inhibitors of MEK1/2 counteracted the loss in GJC elicited by menadione. Like menadione, the chemotherapeutic quinone doxorubicin activated ERK1/2, resulting in connexin phosphorylation and in the decrease in GJC. This is of interest for chemotherapeutic approaches exploiting the bystander-effect which is based upon intact GJC. In fact, cytotoxicity of doxorubicin was elevated in the presence of U0126, an inhibitor of MEK1/2, which blocked doxorubicin-dependent connexin phosphorylation.

The zinc-finger domains of kinases function as redox sensors

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Whereas the classical kinase signal network is universally regarded as an ubiquitous tool that cells employ for intracellular and intracellular communication, the existence of a redox signaling network has not yet found broad acceptance by cell biologists. Many examples of redox regulated signal molecules, including receptor tyrosine kinases and phosphatases, make it abundantly clear that redox related signaling processes, dating from primordial evolutionary times, are active in higher eucaryotes. We show that a number of serine/threonine kinases are dual-responsive, using classical signals and redox reactions as alternate activation cues. Both pathways converge on the zinc-finger domains embedded in the regulatory domains of protein kinase C and cRaf. We show that redox activation is catalyzed by vitamin A that binds to a specific site in the zinc finger domain. These regions act as redox sensors. Oxidation converts thiol groups to disulfide with consequent mobilization of Zn^{2+} ions, an implicit hinge-like opening of the zinc-finger structure and demonstrable catalytic capability. Of note, the activation via the classical second messenger pathway by diacyl glycerol or phorbol ester also causes zinc mobilization. Thus, zinc-finger domains of kinases represent major nodes of cross-communication between the redox and the phosphorylation networks.

4-hydroxynonenal and age precursors impair tyrosine kinase receptor activity

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Reactive carbonyl compounds (RCCs), formed during the oxidation of polyunsaturated fatty acids or carbohydrates, are the precursors of advanced lipoxidation end products (ALE) and advanced glycation end products (AGE) which are found in tissue proteins during ageing and pathologies such as diabetes, atherosclerosis or neurodegenerative disorders. RCCs are able to modify proteins by reacting with lysine, arginine or thiols residues, thereby forming RCC-adducts, which alter protein structure and function. We and others have recently reported that tyrosine kinase receptors (RTKs) can be derivatized by RCCs such as 4-hydroxynonenal (4-HNE, a lipid peroxidation aldehyde present in oxidized LDL) or AGE precursors (glyoxal and methylglyoxal) in vascular cultured endothelial and smooth muscle cells. RTKs modification (EGF and PDGF receptors) by RCCs is dependent on aldehyde concentration and on the time of contact with the cells, and is followed by a progressive alteration of their function (tyrosine kinase) and subsequent signaling. Low RCCs concentrations activate rapidly RTKs phosphorylation and subsequent signaling including SMC proliferation, whereas higher concentrations are inhibitory and impair cell survival and proliferation. The formation of RCC-adducts on RTKs is antioxidant-insensitive, but can be inhibited by carbonyl scavengers (aminoguanidine, hydrazine derivatives), that restore a normal signaling response to ligand stimulation. The presence *in vivo* of HNE-adducts on RTKs isolated from aortas of hypercholesterolemic animals, suggests that these compounds represent more than simple oxidative stress markers and a should play a role in the pathophysiology of oxidative stress-related diseases such as atherosclerosis.

Proinflammatory and proapoptotic effects of oxysterols

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Hypercholesterolemia is certainly associated to progression of atherosclerosis, but the mechanisms by which a relatively inert molecule such cholesterol contributes to the disease process are still quite undefined. Oxysterols, namely 27 carbon atom oxidation products of cholesterol, are by far more reactive than the parent compound. Their role in atherosclerosis is at present under investigation. When the oxysterols more represented in LDL of hypercholesterolemic patients, were simultaneously added to human promonocytic cells (U937), at concentrations of pathophysiologic interest, a significant stimulation of MCP-1 and MIP-1α expression was consistently achieved, without any change in cell viability. Of note, the single oxysterols, when cells were challenged with their relative concentration in the mixture, did not show any significant effect on the expression of these two genes. Also when the mixture was replaced by equimolar concentrations of unoxidised cholesterol, no gene modulation was detectable. Finally, among the oxysterols considered in the study, 7-ketocholesterol, known to accumulate in atherosclerotic plaques, showed a marked proapoptotic effect, when employed alone, at the same concentration of the mixture. *Conclusions*: a mixture of oxysterols compatible with those found in LDL is able, ulike unoxidised cholesterol, to exert proinflammatory effect by up-regulating MCP-1 and MIP-1α expression in a human monocytic cell line, without inducing cell death. When defined oxysterols, in particular 7-ketocholesterol, reach a critical concentration within the atheroma, a proapoptotic effect may support the proinflammatory one.

1-cysPeroxiredoxin as a lung antioxidant enzyme

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Peroxiredoxins (Prx) are a recently described and widely distributed family of peroxidases. Of the 6 mammalian isoforms, 5 use thioredoxin as the redox cofactor while 1-cysPrx uses GSH. 1-cysPrx has a single conserved cysteine and forms a sulfenic acid as the redox intermediate. The enzyme shows broad substrate specificity for hydroperoxides ranging from H_2O_2 to phosphatidylcholine hydroperoxide with similar kinetic constant of approximately 2-5x 10^6 M^{-1} sec^{-1}. 1-cysPrx is widely expressed in mammalian tissues and is especially enriched in lungs, brain, eye, and testes. Exposure of rats or mice to 100% O_2 for 48-63 h resulted in an approximate doubling of lung 1-cysPrx mRNA, protein, and activity suggesting an anti-oxidant role for the enzyme. To test its role in anti-oxidant defense, we used a lung epithelial cell line (H441) to stably overexpress 1-cysPrx as a fusion protein with green fluorescent protein. Overexpressing cells showed increased resistance to Cu-ascorbate-generated oxidative stress with decreased accumulation of lipid peroxides and less annexin V binding. In another lung epithelial cell line (L2), antisense treatment reduced 1-cysPrx expression and resulted in lipid peroxidation and cellular apoptosis as indicated by increased annexin V binding and TUNEL assay. These results indicate that 1-cysPrx can function as an antioxidant enzyme and can protect cells from oxidant-mediated injury by oxidant scavenging and reduction of peroxidized membrane phospholipids.

Refs.: Fisher, A.B. et al. JBC **274:**21326, 1999. Chen, J-W. et al. JBC **276:** 28421, 2000. Manevich, Y. et al. PNAS **99:**11599, 2002. Pak, J-H. et al. JBC **277:**49927, 2002

Novel functions of the mammalian cytosolic and mitochondrial thioredoxin reductases

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Thioredoxin (Trx) is a small ubiquitous protein (12 kDa) that is conserved in all organisms from lower prokaryotes to human and functions as a general protein disulfide reductase. The redox activity of thioredoxin resides in the sequence of its conserved active site Cys-Gly-Pro-Cys (CGPC), which undergoes reversible oxidation of the two cysteine residues from a dithiol to a disulfide form. Thioredoxin is maintained in its active reduced form by the flavoenzyme thioredoxin reductase (TrxR), a multifunctional selenocysteine containing oxidoreductase that uses the reducing power of NADPH (the thioredoxin system).

Recently, we reported the cloning of a mammalian mitochondrial thioredoxin system, (Trx2 and TrxR2). To investigate its biological role, we have generated HEK-293 cells overexpressing either Trx2 or TrxR2. Using these cell lines, we showed that overexpression of Trx2 confers resistance towards etoposide induced cytotoxicity and leads to interference with ATP synthase activity. Furthermore, we demonstrated that cells overexpressing Trx2 have an increased mitochondrial membrane potential ($\Delta\psi_m$). Using our TrxR2 cells we showed that TrxR2 interacts with specific components of the mitochondrial respiratory chain. Taken together, our results suggest that mitochondrial thioredoxin plays a significant role in mitochondrial function and is involved in the regulation of the mitochondrial death pathway.

Furthermore, we constructed stable cell lines that expressed active cytosolic thioredoxin reductase (TrxR1) and we show that TrxR1 could be a very efficient ubiquinone reductase. Finally, we will present evidence that TrxR1 reacts with specific proteins and is translocated to the nucleus.

Thioredoxin Binding Protein TBP-2/VDUP1: Involvement in Cell Cycle and Aging

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Thioredoxin (TRX) is one of the vital components of the thiol reducing system and plays multiple roles in cellular processes such as gene expression, cell proliferation and apoptosis. TRX expression is induced by a variety of oxidative stimuli, including UV irradiation, inflammatory cytokines or chemical carcinogens. We have identified TRX binding protein-2 (TBP-2), identical to vitamin D3 up-regulated protein 1 (VDUP1) as a negative regulator of TRX on its expression and reducing activity (1).

Expression of TBP-2 is lost in HTLV-I-positive IL-2-independent T-cell lines, but maintained in HTLV-I-positive IL-2-dependent T-cell lines as well as HTLV-I-negative T-cell lines. Ectopic overexpression of TBP-2 in HTLV-I-positive T-cells resulted in growth suppression accompanied by a decrease of TRX reducing activity. In the TBP-2-overexpressing cells, G1 arrest was observed in association with an increase of p16 expression and reduction of Rb phosphorylation. The results suggest that TBP-2 plays a crucial role in the growth regulation of T-cells through the interaction with its target molecules including TRX. Therefore, loss of TBP-2 expression may be involved in the multistep oncogenic process of HTLV-I transformation. The modulation of TRX functions may be a new therapeutic strategy for the treatment of HTLV-I related diseases (2).

We have shown that TRX overexpressed mice show a significant resistancy against variety of oxidative stresses, showing increased longevity (3). As TBP-2 is a negative regulator of TRX, we

hypothesize that TBP-2 expression is involved in the aging process as well. The TRX-TBP-2 interaction may be an important redox regulatory mechanism in cellular aging processes.

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SESSION II
MITOCHONDRIA

Characteristics of channel formation in the ADP/ATP carrier: Implications for the mitochondrial pore transition

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The involvement of the ADP/ATP carrier (AAC) in the mitochondrial pore transition is studied on AAC isolated from mitochondria (mAAC) and on recombinant AAC expressed in E.coli (rAAC) by patch clamp measurements on single channels. rAAC has the advantage over mAAC that no traces of other mitochondrial components are carried along such as VDAC or cyclophilin which may modulate the AAC response. Essential features of Ca^{++} induced high conductance first shown with mAAC were similar with rAAC. These are Ca^{++} dependence, high voltage gating , inhibition by ADP and bongkrekate . In addition, the influence of cyclophilin on the currents identified cyclophilin as a regulator of the voltage gating rather than obligatory channel adjunct. The activation by peroxides was shown to be due to a block of the voltage gating machinery reflecting the outstanding sensitivity of the AAC to ROS.A physiologically important asymmetry of voltage gating was demonstrated as it requires high voltage (>140 mV) only from the cytosolic side. The implications of these findings for the mitochondrial pore transition will be discussed taking into account the particular sensitivity of the AAC – cardiolipin complex to Ca^{++} and ROS.

The mitochondrial permeability transition: From molecular mechanism to cardiac protection

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The Mitochondrial Permeability Transition Pore (MPTP) is a non-specific pore that opens in the inner mitochondrial membrane under conditions of elevated matrix [Ca^{2+}], especially when this is accompanied by oxidative stress and depleted adenine nucleotides. Extensive work from this and other laboratories has led to the proposal that opening of the MPTP involves a calcium-mediated conformational change of the adenine nucleotide translocase (ANT), facilitated by bound cyclophilin-D (CyP-D). Cyp-D is the target of two potent inhibitors of the MPTP, cyclosporin A (CsA) and sanglifehrin A (SfA). Adenine nucleotides inhibit MPTP opening by binding to the ANT, and this is antagonosed by oxidation of critical thiol groups on the ANT that greatly sensitise the MPTP to [Ca^{2+}]. In the perfused heart we have demonstrated that MPTP opening does not occur in ischaemia itself, but during subsequent reperfusion when a burst of ROS occurs. Subsequently, the MPTP can close again and the extent of this correlates with functional recovery of the heart. Hearts can be protected from reperfusion injury by direct inhibition of the MPTP with CsA and SfA and by other protocols such as ischaemic preconditioning and treatment with propofol and pyruvate prior to ischaemia that also decrease MPTP opening by an indirect mechanism. In collaboration with cardiac surgeons we are refining these protocols for use in open heart surgery and the treatment of coronary thrombosis.

Mitochondria in nitric oxide-induced cell death

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Nitric oxide (NO) from inducible NO synthase (iNOS) mediates cell death in a wide range of inflammatory, infectious and neurodegenerative pathologies. We find that 2 main mechanisms are involved: (a) energy depletion-induced necrosis (preventable by active glycolysis), and (b) oxidant-induced apoptosis (preventable by antioxidants or caspase inhibitors). NO and its derivatives have 3 relevant actions on mitochondria: (i) inhibition of mitochondrial respiration, reversibly at complex IV, irreversibly at other sites; (ii) induction of mitochondrial oxidant production (O_2^-, H_2O_2, $ONOO^-$, NO_2, N_2O_3), and (iii) induction of permeability transition.

Neurons are uniquely sensitive to NO, due to NO inhibition of mitochondrial respiration inducing rapid glutamate release from neurons, followed by excitotoxic death. Activated astrocytes and microglia potently, rapidly and selectively kill co-cultured neurons, and such death is prevented by iNOS inhibitors and a NMDA receptor antagonist, and is replicated by authentic NO or NO donors. Activated glia maintain 0.5 μM extracellular NO, and immediately inhibit the respiration of co-incubated neurons, resulting in ATP depletion and glutamate release. NO also caused rapid calcium-dependent exocytosis of vesicular glutamate (and ATP) from astrocytes. All these reactions are likely to contribute to the inflammatory neurodegeneration that accompanies many CNS pathologies, including Alzheimer's disease and stroke.

Bal-Price, A. & Brown, G. C. (2001) Inflammatory neurodegeneration mediated by nitric oxide from activated glia, inhibiting neuronal respiration, causing glutamate release and excitoxicity. J. Neuroscience 21, 6480-6491.

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Regulation of the Pyruvate Dehydrogenase Complex by R-lipoic acid

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R-Lipoic acid (R-LA) serves as a prosthetic group of several enzymes involved in mitochondrial metabolism. R-LA is known as an antioxidant and has recently been shown to increase glucose uptake and to lower serum lactate and pyruvate levels in diabetic subjects by activating insulin-signaling pathway. We have investigated whether pyruvate oxidation is increased by R-LA by modulating pyruvate dehydrogenase complex (PDC) activity through its regulation by phosphorylation. Four pyruvate dehydrogenase kinase (PDK) isoenzymes present in mammalian tissues regulate activity of the PDC by phosphorylation of its pyruvate dehydrogenase (E1) component. R-LA and S-LA inhibited four PDKs reconstituted in PDC to different extents. Both R-LA and its reduced form caused inhibition of PDKs: PDK1 > PDK4 ~ PDK2 > PDK3. Phosphorylation of sites 1, 2 and 3 of E1 by PDK1 was reduced to the same extent by R-LA. Since lipoic compounds inhibited PDK activities in the presence of E1 alone, dissociation of PDK from the lipoyl domains of E2 is not a likely explanation for observed inhibition. The reduction in autophosphorylation of PDK2 caused by R-LA indicated that lipoic compounds exerted their inhibitory effect on PDKs directly. An inhibitory effect of R-LA on PDKs would result in less phosphorylation of E1 and hence increased PDC activity. This finding is consistent with the observed glucose lowering effect of R-LA in diabetic subjects and usage of R-LA as a therapeutic agent in the treatment of diabetic neuropathy.

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Mitochondrial nitric oxide synthase and redox signaling

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It is noteworthy that changes in the expression and activity of constitutive mtNOS will be followed by significant variations in matrix NO steady-state levels in the relatively small and well-differentiated mitochondrial compartment (Giulivi et al, 1998). The mitochondrial utilization of NO involves the production of superoxide anion and hydrogen peroxide (H_2O_2) a species freely diffusible outside the mitochondria (Poderoso et al, 1996 and 1999, Antunez and Cadenas, 2000). In the last years, cumulative evidence showed that H_2O_2 production and the consequent oxidative stress level play an important role in the activation of signaling molecules which control the complex machinery involved in cell proliferation, differentiation, apoptosis, senescence, cell transformation and cancer (Davies 2000, Huang et al, 2000). On this basis, it was interesting to analyze the modulation of mtNOS in the frame of cellular redox state and cell cycle progression. These variables were then followed in normal quiescent tissues, in proliferating ones, in tumoral tissues and in transformed cell lines. Collectively, the data show that a) normal proliferating tissues like developing brain or liver have substantially less mtNOS content and mitochondrial H_2O_2 yield than quiescent non-proliferating tissues b) transition of proliferation to tissue differentiation involves a relatively rapid mtNOS increase. Conversely, liver proliferation induced by a single T4 dose is followed by a marked and transient decrease of mtNOS. In addition, tumoral cells exhibit very low mtNOS activity and H_2O_2. Therefore, cascades like D cyclins and MAPKs responding to H_2O_2 result ultimately modulated by changes in mtNOS, which may constitute a platform contributing to persistent cell proliferation or to cell cycle arrest.

Oxidative stress & brain mitochondrial function

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Oxidative stress and loss of brain mitochondrial function has been implicated in the pathogenesis of a number of neurological disorders such as Parkinson's disease, Alzheimer's disease and multiple sclerosis. Furthermore, in such disorders there is now evidence to suggest that neuronal damage can arise as a result of the production and liberation of oxidising species from astrocytes. Certainly, cell culture studies demonstrate that astrocyte-derived nitric oxide can, under certain conditions, cause persistent damage to the neuronal mitochondrial electron transport chain particularly at the level of cytochrome oxidase. However, it now appears that initially the degree of damage inflicted is limited by the neuronal antioxidant capacity, which in turn is influenced by the presence of astrocytes. Thus, astrocytes appear to have the capacity to release reduced glutathione (GSH) that is subsequently utilised by the neurone to enhance the intracellular concentration of GSH. In addition to having the ability to release GSH, astrocytes also release a factor, possibly the extracellular isoform of superoxide dismutase, that retards the oxidation of GSH in the extracellular environment and so ensuring optimum utilisation be the neurone [**1**]. Consequently, perturbation of the trafficking of GSH precursors from astrocyte to neuron could render the neuronal mitochondrial electron transport chain susceptible to oxidative damage leading ultimately to neuronal cell death.

[**1**]. J. Neurochem. (2002), 83, 984-991.

Mitochondria, proteolysis, calcium, and apoptosis

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Mitochondrial aconitase is sensitive to oxidative inactivation and can aggregate and accumulate in various age-related disorders. We now report that the ATP-stimulated, mitochondrial-matrix, "Lon" protease selectively recognizes and degrades the oxidized, hydrophobic form of aconitase after mild oxidative modification but severe oxidation results in aconitase aggregation, which makes it a poor Lon substrate. Similarly, a morpholino oligodeoxynucleotide directed against the *lon* gene dramatically decreased Lon protein levels, Lon activity, and aconitase degradation in WI-38 VA-13 human lung fibroblasts, and caused accumulation of oxidatively modified aconitase. The ATP-stimulated Lon protease may be an essential defense against the stress of life in an oxygen environment. By recognizing minor oxidative changes to aconitase structure, and rapidly degrading the mildly modified protein, the Lon protease may prevent extensive oxidation, aggregation, and accumulation, which could otherwise compromise mitochondrial function and cellular viability. Aconitase is probably only one of many mitochondrial matrix proteins that are preferentially degraded by Lon following oxidative modification.

We compared Lon protease expression in murine skeletal muscle from old and heterozygous (*Sod2*$^{-/+}$) mice, and studied Lon involvement in the accumulation of damaged (oxidized) proteins. Lon protease protein levels were lower in old and oxidatively challenged animals. Lon deficiency was associated with increased levels of carbonylated proteins. We identified one of these proteins as aconitase, and another as an aconitase fragmentation product, which we can also generate in vitro by treating purified aconitase with H_2O_2. These results imply that aging and oxidative stress downregulate Lon protease expression, which, in turn, may be responsible for the accumulation of damaged proteins (aconitase) within mitochondria.

Mitochondrial Lon is a major controller of multiple mitochondrial functions: regulates the accumulation and degradation of oxidatively-modified matrix proteins, chaperones the assembly of inner membrane complexes, and participates in the maintenance of mitochondrial DNA. *Lon* down-regulation in WI-38 VA-13 human lung fibroblasts elicited two temporally and mechanistically distinctive patterns. Initially, *lon* deficient fibroblasts underwent massive, calcium-dependent, caspase 3 activation and apoptotic death. Unlike oxidative stress-induced apoptosis, however, widespread calcium-dependent degradation of mitochondrial RNA and DNA was **not** observed during the early stages of apoptosis induced by *lon* deficiency. At a later stage, the surviving cells failed to divide, displayed highly abnormal mitochondrial function and morphology and essentially reverted to an anaerobic metabolism. Mitochondrial mass decreased as result of mitochondrial inability to divide. Our results indicate that while the proteolytic and chaperone activity of Lon are mostly involved in the early stage of *lon* deficiency, all three of its functions are affected at latter stages of *lon* deficiency.

Mitochondria and signaling lipids in apoptosis

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The interaction of proteins with mitochondria sets in motion molecular pathways leading to cell death. In addition to the proapoptotic function of proteins, the physical interaction of lipids with mitochondria is emerging as a novel process in death ligand-induced cell death. Our data indicate that the formation of ceramide from acidic sphingomyelinase (ASMase) contributes to TNF-α-mediated hepatocellular death. This effect of ASMase is exerted by ceramide-stimulated ganglioside formation as prevention of glycosphingolipid synthesis protected sensitized hepatocytes against TNF-α-induced cell death despite overproduction of ceramide levels. In examining the distribution of ganglioside GD3 (GD3) in hepatocytes we observed a redistribution of GD3 from the plasma membrane in response to TNF-α colocalizing with mitochondria as revealed by immunoelectron and scanning confocal microscopy. The targeting of GD3 with mitochondria was prevented by actin filaments-disrupting agents and by sequestering plasma membrane cholesterol at caveolae. Even though GD3 undergoes a redistribution and subsequent targeting from the plasma membrane to mitochondria in response to various apoptotic stimuli, the consequences of this interaction are controlled by the levels and availability of mitochondrial glutathione (mGSH). Thus, selective mGSH depletion with the sparing of cytosolic GSH sensitized hepatocytes to TNF-α-induced apoptosis that was preceded by mitochondrial membrane depolarization, cytochrome c release and caspase activation. The mitochondrial uptake of GSH was dependent on appropriate membrane fluidity range and mitochondrial cholesterol enrichment impaired selectively the mitochondrial uptake of GSH resulting in reduced mGSH levels. Chronic alcohol intake stimulated the levels of cholesterol through endoplasmic reticulum stress sec-

ondary to acetaldehyde formation and the targeting of cholesterol into mitochondria resulting in lower mGSH levels sensitized hepatocytes to TNF-α-induced death. Thus, in addition to the proapoptotic role of proteins, the targeting of cholesterol and GD3 to mitochondria regulate different features of apoptosis by promoting depletion of mGSH levels and stimulation of mitochondrial reactive oxygen species generation.

Mitochondrial regulation of apoptotic cell death

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Although it has long been known that impairment of mitochondrial function may lead to ATP depletion and necrotic cell death, recent work has revealed that these organelles also play an important role in the overall regulation of apoptotic cell death by mechanisms which have been conserved through evolution. Thus, it seems that a number of death triggers target the mitochondria and stimulate their release of cytochrome c and other pro-apoptotic proteins, which can trigger caspase activation and other parts of the apoptotic process. Cytochrome c release is governed by the Bcl-2 family of proteins, whereas subsequent caspase activation is modulated by other proteins, including inhibitor of apoptosis proteins (IAPs) and heat shock proteins. Recent findings indicate that cytochrome c extrusion occurs by a two-step process, which is initiated by a disruption of the association of this protein with cardiolipin, the phospholipid that anchors it to the outer surface of the inner mitochondrial membrane. Release of the solubilized pool of cytochrome c into the cytosol may then occur by pore formation mediated by pro-apoptotic Bcl-2 family proteins, notably Bax and Bak, or by mitochondrial permeability transition followed by matrix swelling and rupture of the outer mitochondrial membrane. Recent evidence suggests that cytochrome c release during apoptosis may in fact involve a combination of these two mechanisms. Taken together, these findings have placed the mitochondria in the focus of apoptosis research and further underlined the important function of these organelles in cell life and death.

SESION III
AGING AND NEURODEGENERATION

NADH-dehydrogenase as a marker of aging

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NADH-dehydrogenase (Complex I) is a membrane bound flavoprotein (about 900 kDa, at least 42 polypeptides) that channels reducing equivalents from the matrix to the mitochondrial respiratory chain. This protein complex was early recognized as highly sensitive to amphiphilic molecules such as barbiturates, steroids, fatty acids and lysophospholipids. Peroxynitrite inhibits NADH-dehydrogenase in liver and heart mitochondria, and selective inhibition of brain NADH-dehydrogenase has been reported in Parkinson disease and MPTP-induced parkinsonism. Brain mitochondrial NADH-cytochrome c reductase activity was selectively decreased upon aging. Activities (in nmol NADH/min/mg protein), at 28, 52 and 72 wk of mice age, were: 387 ± 26, 348 ± 26, and 225 ± 25 in males and 394 ± 22, 342 ± 28, and 268 ± 25 in females (with 10-13% and 34-42% decreased activities at 52 and 72 wk). Succinate-cytochrome c reductase activity was unchanged. Decreased NADH-dehydrogenase activity associated to unchanged cytochrome c reductase activity is interpreted as a selective loss of NADH-dehydrogenase activity. Cytochrome oxidase activity, another marker of mitochondrial aging, was 14-18% and 33-37% decreased at 52 and 72 wk of age (with respect to 28 wk). Liver mitochondrial NADH-cytochrome c reductase activity was similarly decreased upon aging. Activities (in nmol NADH/min/mg prot.), at 28, 52 and 72 wk of mice age, were: 436 ± 21, 348 ± 16, and 240 ± 12 in males and 494 ± 20, 374 ± 19, and 292 ± 22 in females (with 20-24% and 41-45% decreased activities at 52 and 72 wk). Succinate cytochrome c reductase activity was unchanged, and cytochrome oxidase activity was 11-23% and 17-44% decreased at 52 and 72 wk of age (with respect to 28 wk). NADH-cytochrome c reductase activity was also selectively decreased in heart and kidney mitochondria upon aging. NADH-cytochrome c reductase activity statistically and inversely correlated with TBARS levels and pro-

tein carbonyls in mitochondrial membranes in brain, liver, heart, and kidney. Brain NADH dehydrogenase activities statistically correlated with mice success levels in behavioral tests, such as tight-rope movements and T-maze exploratory activities. NADH-dehydrogenase activity appears as a marker of mouse aging, likely due to the cumulative oxidative effects of peroxynitrite, peroxyl radicals and hydroperoxides.

Mitochondrial reactive oxygen species production in aging: Gender differences and redox sensitive signaling pathways

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According to the free radical theory of aging, oxygen free radicals are responsible for aging and for the occurrence of age-associated diseases. Mitochondria are a major source of free radicals in cells. Recently, an inverse relationship between the rate of mitochondrial free radical production and the maximum life span of mammalian species has been found. In the present study, we have investigated the differential mitochondrial oxidative stress between males and females to understand the molecular mechanisms enabling females to live longer than males.

Liver or brain mitochondria from female rats generate approximately half the amount of peroxides produced by those of males. This is completely reversed by ovariectomy. Estrogen replacement therapy prevents the effect of ovariectomy on free radical production by mitochondria. Oxidative damage to mitochondrial DNA in males is four fold higher than that of females. To explain these facts, we measured reduced glutathione levels as well as the expression and activities of superoxide dismutase and glutathione peroxidase. They are higher in mitochondria from females that in those from males. Moreover, 16S rRNA expression, which decreases significantly with aging, is four times higher in mitochondria from females than in those from males of the same chronological age.

Mitogen-activated protein (MAP) kinase signaling cascades are induced by stress stimuli, such as oxidative stress, mediating most their effects on cell death. The effect of ß-estradiol on the activation of MAP kinases has been assessed.

The present study provides molecular evidence to explain the different life span in males and females.

Implication of inflammation in the degeneration of nigral dopaminergic system

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The loss of dopaminergic neurons in the substantia nigra (SN) is a hallmark of Parkinson's disease (PD) but the etiology of the disease is still mainly unknown. The dramatic proliferation of reactive amoeboid macrophages and microglia seen in SN of PD brain together with oxidative stress, allow to suggest that immunemediated events along with the inductions of inflammatory process could be involved in the dopaminergic neurodegeneration. We have studied the effects produced by the induction of the inflammatory process in the dopaminergic neurodegeneration afterwards we have also studied its inhibition on this process. First at all, we have studied the single injection of the immunostimulant lipopolisacharide (LPS) in different areas of the SNC. LPS induced a strong macrophage/microglial reaction in SN, with a characteristic clustering of macrophage cells around blood vessels. The SN was far more sensitive that striatum to the inflammatory stimulus. Moreover, only the dopaminergic neurons of the SN were affected, with no detectable damage to either the GABAergic or the serotoninergic neurons. The damage to the DA neurons in the SN was permanent, as observed 1 year postinjection. These results support that LPS cause an indirect neuron death due to inflammatory reaction. These are also supported by the fact that this dopaminergic neurodegenerative process produced by LPS was prevents by dexamethasone treatment. Moreover, some physiological inductor of inflammatory process could be also involved in this neurodegenerative process. It is known that thrombin is also able to induce the activation of culture rodent microglia. Thrombin produced a strong macrophage/microglial reaction in SN with the induction of iNOS, Il-1β and TNF-α along with a selective damage to dopaminergic neurons. All these results strongly support the implication of the inflammatory process in the neurodegeneración of the dopaminergic neurons in SN.

The beneficial effects of fruit polyphenols in brain aging may involve more than antioxidant activity

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Dietary supplementation (S) with fruit or vegetable extracts high in antioxidants (e.g., blueberry, BB, spinach) can decrease the enhanced vulnerability to oxidative stress (OS) that occurs in aging and these reductions are expressed as improvements in neuronal signaling and behavior. In addition, recent examinations using striatal or hippocampal tissue isolated from BB supplemented aged animals, have shown that striatal slices show reductions in H2O2 - induced decrements in muscarinic receptor sensitivity decrements, while the hippocampal slices show decreases in baseline levels of HSP-70 and increases in HSP-70 responsiveness to lipopolysaccharide (LPS) exposure. Moreover, there are also indications that BB supplementation can also reduce the sensitivity to neurotoxic agents (kainic acid) that induce oxidative stress and inflammation. Additional experiments suggest that BB effects also may include enhancement of neuronal signaling. Therefore, it appears that polyphenolic compounds such as those found in BB may exert their beneficial effects by enhancing the endogenous anti-inflammatory, antioxidant and neuronal signaling capabilities of the organism. However, recent work from our laboratory has indicated that one of the most striking effects of BB supplementation may involve increases in neurogenesis. It is known that factors such as head injury, depression and stress that lead to decreases in neurogenesis are all associated with greater rates of cognitive decline. Conversely, exercise and environmental enrichment can improve both neurogenesis and cognitive function in aging. The results of our latest study has indicated that aged BB-supplemented rats, tested in the radial arm water maze (RAWM) and given injections of BrdU showed that the number of proliferating cells in the dentate gyrus were significantly higher ($p < 0.05$) in the BB-fed rats. Moreover,

these findings were correlated with improvements in the RAWM performance such that as the number of proliferated cells increased, the number of memory errors decreased (reference memory errors: $r = - 0.654$, $p < .05$, working memory errors: $r = -0.646$, $p < .05$, total memory errors: $r = -0.587$, $p = .08$). These findings suggest that antioxidant-rich fruits such as BBs may improve cognitive performance in aged animals by increasing proliferation of neural precursor cells in the hippocampus.

Cell signaling and apoptosis in aging

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There is strong evidence that mitochondrial dysfunction *in vivo* plays a significant part in the pathogenesis of neurological disorders and sarcopenia (atrophy and loss of skeletal muscle myofibers) (1-3). Life-long caloric restriction (CR) has been shown to have neuroprotective effects, prevents the age-associated loss in muscle fibers and function, but the mechanisms *in vivo* are unknown. We investigated apoptosis and apoptotic regulatory proteins in skeletal muscle and the brain frontal cortex of 12-month old, 26-month old *ad libitum* fed and 26-month old CR male Fischer-344 rats (CR = 40% of *ad lib* levels). We found that specific DNA fragmentation, indicative of apoptosis, was increased with age in these two post-mitotic tissues and that CR attenuated this age-associated increase significantly (4,5). We determined levels of ARC (apoptosis repressor with a caspase recruitment domain), which inhibits caspase-2 activity and attenuates cytochrome c release from the mitochondria in addition to levels of XIAP (X-linked inhibitor-of-apoptosis), which inhibits caspase-3 activity. We found a significant age-associated decline in ARC levels, which were attenuated by CR. In accordance with the changes in ARC expression in the brain, CR attenuated the increases in cytosolic cytochrome c and caspase-2 activity observed during aging (5). Moreover, we found that CR suppressed the age-associated rise in cleaved caspase-3 in skeletal muscle and in the cerebral cortices. XIAP protein content increased with age and was reduced by CR in both tissues. Our studies demonstrate that post-mitotic tissues show significant alterations in apoptotic signalling with aging and that caloric restriction is able to modulate these changes.

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Caloric restriction, gene expression, and aging

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To provide a global analysis of gene expression in the aging heart, we monitored the expression of 9,977 genes simultaneously in 5- and 30-month-old male B6C3F(1) mice by using high-density oligonucleotide microarrays and several statistical techniques. Aging was associated with transcriptional alterations consistent with a metabolic shift from fatty acid to carbohydrate metabolism, increased expression of extracellular matrix genes, and reduced protein synthesis. Caloric restriction (CR) started at 14 months of age resulted in a 19% global inhibition of age-related changes in gene expression. We have also determined the gene expression profile associated with acute oxidative stress in the heart of young and old C57Bl6J mice, and have discovered specific age-related alterations in this transcriptional response. Our observations provide evidence that aging of the heart is associated withspecific transcriptional alterations, and that CR initiated in middle age may retard heart aging by inducing a profound transcriptional reprogramming.

Mechanism of action of antioxidants cDNA microarray gene expression

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Objective: Significant evidence has been provided to support the hypothesis that oxidant stress may be responsible for degeneration of dopaminergic neurons in the substantia nigra pars compacta in Parkinson's disease. Dopamine (DA), R-apomorphine (R-APO, a dopamine D_1/D_2 receptor agonist), green tea polyphenol (-)-epigallocatechine-3-gallate (EGCG) and melatonin are neuroprotective and radical scavenger compounds. The aim of our study was to establish the mechanism of the concentration-dependent neuroprotective and pro- apoptotic action of theses drugs via gene expression and protein determination.

Methods: cDNA microarrays provide new prospects to study and identify various mechanisms of drug action. We have employed this technique for the present study. Total RNA was extracted from SH-SY5Y cells exposed to low neuroprotective and high toxic concentrations of the drugs, followed by synthesis of first strand cDNA and hybridization to a cDNA expression array membrane related to apoptosis, survival and cell cycle pathways. The results were confirmed by quantitative real-time PCR. Protein profiles were assessed by 12% SDS gel electrophoresis, following by western blotting using chemiluminescence's method.

Results: High concentration of both DA (500μM) and R-APO (50μM) exhibited a similar profile of expression at the two time intervals tested (1.5hr and 6hr) increasing bax, caspase3, caspase6, fas ligand and the cell-cycle inhibitor gadd45 while decreased anti-apoptotic Bcl-2 and Bcl-X. However, melatonin affected only Gadd45 at the short exposure time (1.5hr). At the low protective concentration, DA (10μM), R-APO (1μM) and EGCG (1μM) decreased the expression of Bax , Bad and Gadd45, while Bcl-2 and Bcl-X mRNA were decreased only by DA and R-APO.

Conclusions: Our results have provided for the first time new insights into the gene mechanisms involve in both the neuroprotective and pro-apoptotic activities of neuroprotective drugs. We have shown that DA and R-APO demonstrated similar patterns of gene expression and protein profiles, while EGCG and melatonin displayed their own gene and protein expression pattern. These results suggest that different anti-oxidant drugs may exert neuroprotective/ pro-apoptotic activity by specific cell pathways.

SESSION IV
NITRIC OXIDE, CELL SIGNALING, AND GENE EXPRESSION

Selective mesurement of nitric oxide production evoked by glutamate agonists in rat hippocampal brain slices

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Nitric oxide (NO•) has emerged as a diffusible signaling radical implicated in a variety of pathophysiological processes. Regarding how the balance between physiology and pathology tilts, a simplistic dogma is currently accepted: NO• acts physiologically at low nmolar range and an increase in NO• concentration shifts the balance towards pathology. Paradoxically, it has not been inequivocally determined what is a physiological and a pathological NO• concentration and the knowledge of NO• concentration dynamics remains rudimentary. This is because the measurement of a diffusible, evanescent and radical molecule such as NO• requires a methodology endowed with "real time", selective, sensitive and spatial resolution characteristics. We have prepared NO• microsensors (8 µm diameter 100 µm tip length), which in connection with amperometry and fast cyclic voltammetry (FCV) meet the above mentioned criteria. Following the concentration dynamics of NO• in a functionally dependent-way on glutamate, NMDA and arginine stimuli in hippocampal brain slices common features for all the stimuli could be identified (*e.g.*, a decrease in peak intensity for sequential stimulations). However, differences in the time-course of •NO dynamics were dependent on the hippocampus functional subregions (*e.g.* CA1 *vs* CA3), thus suggesting different mechanisms of •NO removal/degradation in those areas. The •NO signals could be modulated by dietary phenol caffeic acid. (Supported by FCT).

The knowledge of •NO concentration dynamics remains rudimentary. We have set up an experimental electrochemical approach to measure •NO in a real-time and selective way; using microsensors inserted in rat hippocampal brain slices, we were able to follow the concentration dynamics of •NO in a functionally dependent-way on glutamate, NMDA and arginine stimuli. Whereas

common features for all the stimuli could be identified, such as a decrease in peak intensity for sequential stimulations, differences in the time-course of •NO production and removal/degradation were found, for instance the dependency on the hippocampus CA1 *vs* CA3 region, suggesting different mechanisms of •NO removal/ degradation in those areas. Importantly, •NO signals could be modulated by caffeic acid. This approach may contribute to the elucidation of the mechanisms related with the time course of agonist-induced •NO production in different brain areas.

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Nitric oxide derived oxidants and cardiovascular disease

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Formation of nitric oxide (NO)-derived oxidants may serve as a mechanism linking inflammation to development of atherosclerosis. Nitrotyrosine, a specific marker for protein modification by NO-derived oxidants, is enriched in human atherosclerotic lesions and low density lipoprotein (LDL) recovered from human atheroma. Whether systemic levels of nitrotyrosine can predict risk for coronary artery disease (CAD) and are modulated by therapies know to reduce CAD risk, such as hydroxymethylglutaryl coenzyme A reductase inhibitors (statins), is unknown. To answer these questions, plasma levels of protein-bound nitrotyrosine were determined by mass spectrometry in 333 sequential patients presenting to an academic center Cardiology Department. Nitrotyrosine levels were significantly higher among patients with CAD (median 9.1 umol/mol tyrosine vs. 5.7 umol/mol; $P < 0.001$). Patients in the upper quartile of nitrotyrosine levels had higher risk of CAD relative to the lowest quartile (59% vs. 26%; unadjusted odds ratio, 4.1; 95% confidence interval, 1.9 to 8.5; $P < 0.001$). In multivariate models adjusting for coronary risk factors and C reactive protein, upper quartiles of nitrotyrosine remained predictive for CAD risk (odds ratio, 3.0; 95% confidence interval, 1.3 to 7.1; $P < 0.001$). Statin therapy caused a significant reduction (25%; $P<0.02$) in nitrotyrosine content of plasma proteins that was similar in magnitude to reductions in total cholesterol and LDL particle number (25% and 29%, respectively; $P<0.001$ each), yet independent of alterations in lipoproteins and inflammatory markers like CRP. We conclude that nitrotyrosine, a specific protein modification produced by NO-derived oxidants, serves as a significant and independent marker of CAD, and is modulated by statin therapy. These results support a role for nitric oxide-derived oxidants as inflammatory mediators in CAD and may have important implications for atherosclerosis risk assessment, diagnosis and the monitoring of anti-inflammatory actions of statins.

Nitric Oxide:
The balance between cell viability and apoptotic death

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Nitric oxide plays a relevant role in the regulation of cell viability through the interaction with other reactive intermediates (for example, with superoxide to form peroxynitrite), the expression of genes involved in the control of the apoptotic pathway (for example, some IAPs are up-regulated by sustained moderate concentrations of NO), and through the inhibition of the catalytic activity of caspases and other enzymes that possess critical cysteine residues that may sense NO. Our group has shown that moderate concentrations of NO exert anti-apoptotic effects in various cell types, whereas high-output NO synthesis triggers apoptosis through the engagement of various mechanisms. Cultured macrophages offer a good model system to evaluate the contribution of NO to the cell fate and to the regulation of inflammation under pathophysiological conditions. Activation of macrophages by Gram negative bacteria can be reproduced *in vitro* by incubation with lipopolysaccharide (LPS) and pro-inflammatory cytokines. Under these conditions macrophages participate in the onset of inflammation by releasing cytokines, bioactive lipids (prostaglandins and leukotrienes), reactive oxygen (ROI) and nitrogen intermediates (RNI) and matrix metalloproteinases. At the end of the inflammatory response, the cells that have participated in the process are removed by apoptosis. This apoptosis is mainly due to the elevated synthesis of NO accomplished after the expression of NOS-2, and NO plays a relevant role by altering the expression of genes and the activity of enzymes related to apoptosis, among them the pro-apoptotic members of the Bcl-2 family, IAPs, p53 and the activity of caspases. Finally, this response is important to resolve inflammation and to avoid the establishment of a chronic inflammatory state, characteristic of many common pathologies.

Nitric oxide (NO) mimics hypoxia by accumulating HIF-1α

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Hypoxia inducible factor-1 (HIF-1) is a heterodimer composed of stability regulated HIF-1α and constitutively expressed HIF-1β. HIF-1 binds to HRE (hypoxia response elements) and enhances transcription of hypoxia-inducible genes to orchestrate cellular adaptation to decreased oxygen availability.

Recent evidence suggests that HIF-1α is subjected to stability regulation under normoxia by NO, either liberated by chemically distinct NO donors or generated by iNOS. NO signaling is cGMP-independent but demanded an active phosphotidylinositol-3-kinase/AKT pathway. Mechanistically, we propose that NO interferes with HIF-1α degradation by decreasing its ubiquitination which results from an attenuated pVHL (von Hippel-Lindau protein, a putative E3-ubiquitin ligase)-HIF-1α interaction. This is consistent with an impaired prolyl hydroxylase (HIF-PH) activity which functions as a putative oxygen sensor. As expected, NO-effects are subjected to modulation by superoxide formation with the further notion that the effects of NO is dramatically different under hypoxia, when NO suppressed rather than provoked HIF-1α accumulation.

Our studies imply stabilization of HIF-1α in close association with the formation of NO under normoxic conditions. Thus, HIF-1 apart from regulating the cell response to hypoxia may help to coordinate a proper cell response to inflammation, i.e., NO formation.

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Nitric oxide as a modifier of gene expression: Pathways and mechanisms

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Nitric oxide (NO) is a biological mediator involved in the regulation of a wide variety of functions, including gene expression. The interaction of NO with protein metals, specially the heme group of soluble guanylate cyclase, has been thoroughly studied. Recently, its capacity of interaction with other free radicals and of covalently modifying several protein residues has fostered the interest in understanding the mechanisms by which transcription factor activation/inactivation by NO may occur. Among these posttranslational modifications induced by NO and other reactive nitrogen species (RNS), our interest has focused on the modification of cysteine residues by S-nitrosylation, S-glutathionylation and other oxidative changes.

In previous work we have observed the S-glutathionylation of the recombinant proteins cJun and p50 (components of AP-1 and NF-κB transcription factors, respectively) induced by changes in the redox pair GSH/GSSG and NO in the presence of millimolar concentrations of GSH. These modifications map specifically to one cysteine in each protein, both located in their respective DNA-binding domains. The modifications are associated with an inhibition in their DNA-binding activity and become reversible when the oxidative or nitrosative stress are reduced. Thus, S-glutathionylation could represent a mechanism by which nitrosative and oxidative stress-related signals could be transduced into gene expression changes.

We are currently using different approaches to detect these modifications inside the cell with specific emphasis on their functional relevance. We have developed a method for detecting S-nitrosylation in endothelial cells (in which NO is produced in low quantities in basal conditions) using a proteomic approach. This allowed us to identify several proteins modified by S-nitrosylation.

We are currently trying to exploit the advantage of this method and of other alternative approaches for the detection of this type of modification in transcription factors (such as NF-κB) for which S-nitrosylation has been described and is considered to be of potential regulatory importance.

SESSION V
EXERCISE

Free radicals produced in exhaustive exercise as signals in muscle cell function. Role of xanthine oxidase

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Exercise produces free radicals (1). We found that free radicals are produced only when exercise is exhaustive (2,3). Recently, we postulated that xanthine oxidase is involved in oxidant production in exercise and showed that allopurinol, an inhibitor of this enzyme, prevents damage associated to exhaustion in animals (4).

During the Tour of France 2001, we tested the hypothesis that XO is involved in free radical production in exhaustion. We studied the US Postal Cycling Team which includes the winner of the last four Editions of the Tour. We found that the Team Time Trial stage is the one that causes most muscle damage in cyclists and that allopurinol prevents such damage.

In another line of work, we have studied the role of reactive oxygen species as signals in muscle cell function. We have found that exercise activates the phosporilation of MAP kinases, particularly ERK1 and ERK2 and p38. Furthermore we have found that exhaustive exercise activates NF-kappaB in muscle. The possible relationship between activation of MAP kinases and of NF-kappaB will be discussed. Allopurinol prevents the activation of cell-signalling pathways associated with exercise.

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Melatonin and exercise

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Melatonin functions as a direct free radical scavenger and protects cells from damage by acting cooperatively with other antioxidants. Interventions that reduce the generation or the effects of reactive oxygen species (ROS) exert beneficial effects in a variety of models of inflammation, shock and different pathologies. A situation that results in increased formation of ROS is exercise, in which oxidative damage is often detected within tissues. During prolonged exercise glucose and free fatty acids are utilized as fuels and it is known that a number of different enzymes involved in carbohydrate metabolism are regulated by the redox state of the cell. Specifically, in muscle glucose transport is the rate-limiting step for carbohydrate metabolism and may be influenced by different ROS and reactive nitrogen species. We have studied effects of melatonin treatment on fuel homeostasis in exercised rats. Treadmill exercise to exhaustion resulted in a significant hypoglycaemia and increased plasma levels of lactate and beta-hydroxybutyrate, together with a significant reduction of glycogen in muscle and liver. Those effects were significantly prevented by melatonin, suggesting a preservation of glycogen stores in exercised animals through changes in carbohydrate and lipid metabolism. Administration of the nitric oxide inhibitor L-NAME caused a significant reduction in plasma nitrite and iNOS expression, but liver glycogen and biochemical parameters in blood did not significantly differ from untreated exercised animals, indicating the absence of a direct association between melatonin effects on fuel metabolism and nitric oxide levels. Effects of melatonin during exercise could not only be related to fuel homeostasis but also to their antinflammory properties in part linked to the antioxidants effects of this molecule. Preliminary data obtained in our laboratory suggest that melatonin markedly blocks NFκB activation in exercised muscles, and that this could be related, at least in part, to a significant decrease of oxidative stress.

Contraction-induced oxidants as mediators of muscle adaptations to exercise

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Skeletal muscle generates a number of reactive oxygen and nitrogen species (ROS) during contractile activity. Mitochondria are a major source of intracellular oxidants and there is increasing evidence that muscle cells additionally release ROS into the interstitial space during contractile activity. These extracellular ROS include superoxide (McArdle *et al, Am J. Physiol.* 280: C621-C627, 2001), hydroxyl radicals (O'Neill *et al, J.Appl. Physiol.* 81: 1197-1206, 1996) and nitric oxide (Balon and Nadler, *J. Appl. Physiol.* 77: 2519-2521, 1994). The sources of these oxidants are the subject of current investigation, with extracellular superoxide apparently originating from a plasma membrane-bound oxido-reductase system (McArdle *et al,* 2001). Oxidants appear to be able to stimulate multiple changes in gene expression and our recent data indicated that exposure of muscle cells to exogenous oxidants induced a coordinated transcriptional response to the oxidant. We have also used microdialysis techniques to monitor interstitial ROS *in vivo* (McArdle *et al,* 2001; Pattwell *et al, Free Rad. Biol. Med.* 30: 979-985, 2001) and these have revealed relationships between oxidant generation and specific changes in muscle gene expression that result from contractile activity. These data have implications for understanding the mechanisms of adaptive responses of skeletal muscle to contractile activity and for the use of supplementary antioxidants by athletes.

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Capacity for recovery and possible mechanisms in muscle immobilization atrophy of young and old animals

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Loss of muscle mass with age is a well-known phenomenon called sarcopenia of aging. This phenomenon is virtually universal, found in animals as well as in human beings. A more acute problem is the disuse muscle atrophy caused by injury or immobilization of muscles. We have found that immobilization causes rapid degradation of skeletal muscle proteins and loss of muscle mass, in both young and old rats. Using a model of 4 wk immobilization/4 wk recovery, the recovery capacity of old animals was far slower than that of young ones. Three muscle-degradation systems operate in muscles under various physiologic and pathologic conditions. These are Ca-dependent proteolysis, the lysosomal degradation system, and finally, the ubiquitin (Ub)-proteosome pathway. Our working hypothesis is that one of the major events in muscle atrophy is the infiltration of macrophages and monocytes into degenerating muscle fibers with the increasing secretion of several cytokines such as TNF-α, IL-1, and IL-6. These cytokines, especially TNF-α, increase intracellular oxidative stress, leading to the activation of transcription factors like NF-κB, involved in triggering the Ub-proteasome pathway. Indeed, in our immobilization model the Ub system is implicated as an important factor in muscle degradation. Moreover, NF-κB is known to induce the enzyme inducible nitric oxide synthase, and its involvement in muscle breakdown is also postulated. Elucidating these molecular/cellular events may help in understanding the mechanisms of sarcopenia.

Exercise and NFκB Signaling in Rat Skeletal Muscle

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Nuclear Factor (NF) kB is one of the major oxidative stress-sensitive signal transduction pathways in the mammalian tissues. Activation of NFkB signaling cascade has been shown to enhance the gene expression of several antioxidant enzymes containing NFkB binding sites on DNA, including the mitochondrial superoxide dismutase (MnSOD). We investigated the effect of an acute bout of treadmill running (25 m/min, 5% grade, 1 h) on NFkB signaling and the time course of activation in rat skeletal muscle. NFkB binding to nuclear extracts from deep vastus lateralis (DVL) muscle reached maximal at 1-2 h post exercise, and returned to resting level at 48 h. This was accompanied by an elevated P65 content in DVL. Cytosolic content of IkB, the inhibitory subunit of NFkB complex, was decreased, whereas phosphor-IkB content increased at 0, 1 and 2 h post-exercise. Furthermore, activity of IkB kinase (IKK), the enzyme that phosphorylates IkB, was elevated, as revealed by increased phospho-IKK content in DVL of exercised rats. These data indicate that rigorous exercise activates IKK, leading to IkB phosphorylation and dissociation, and subsequent NFkB nuclear translocation. This cascade may explain the transcriptional activation of MnSOD by exercise reported in our previous study.

SESSION VI
PLANTS

Chlorophyll fluorescence: A reporter of osmotic volume changes and solute/water transport in cyanobacteria

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In cyanobacteria, the supply of electronic excitation by phycobilisomes (PBS) to photosystems II and I (PSII, PSI) is regulated by the state of light acclimation of the cells. Relatively more PBS excitation is apportioned to PSII in light-acclimated (state 1) than in dark acclimated (state 2) cells; also, relatively more PBS excitation is apportioned to PSI in dark-acclimated cells than in light-acclimated cells. As a result, light-acclimated cells emit stronger chlorophyll *a* (Chla) fluorescence (F_1), than dark-acclimated cells (F_2). We have shown that osmotically-induced changes in the volume of the cyanobacterial cell is another factor that regulates Chla fluorescence between levels F_1 and F_2. In fact, osmotic effects on Chla fluorescence override the effects of light acclimation. When cells are shrunk in hyper-osmotic suspension media, the light-induced rise of Chla fluorescence from level F_2 to level F_1 is prevented; conversely, a hyper-osmotic shift suppresses both the volume and the fluorescence of light-acclimated cells. It was, further, established that the magnitude $\Delta F = F_1 - F_2$ is linearly proportional to the osmotically-induced change of the cell volume, and that it obeys the osmotic law of Boyle-van t' Hoff ($\Delta F \quad \Delta V = k\text{Osm}_{OUT}$). A hypothesis about the underlying mechanism of the osmotically-induced changes in the Chla fluorescence of cyanobacteria will be presented, and various applications of the phenomenon will be described, including the determination of cytoplasmic osmolality, rates of water and solute transport across the cell membrane, and thermotropic transition temperatures of cell membrane lipids.

Synergisms in antioxidant function of different classes of natural products

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Plants have to cope with a wealth of abiotic and biotic impacts since some 2-3 billion years. Land plants have to sustain both extreme temperatures or dryness and high light intensities accompanied by oxygen saturation without a chance to escape. In addition to these abiotic impacts infections by virus, bacteria, fungi and wounding by animals, especially insects, may have dramatic effects. Plants thus developed a strategy for defence combining several operative avoidance reactions with a sophisticated set of chemicals, present either constitutively or synthesized „just in time“. Coevolution of animals took advantage of the synthesizing capacity of plants sparing the synthesis of certain „expensive“ groups of chemicals such as aromats (phenolics).

In our days the knowledge about these interrelationships and connections of protective systems is exponentially increasing leading to novel insights mainly concerning medical - pharmacological and nutritive aspects on the following basis: One main common problem during most diseases, both of plants and animals, is „oxidative stress“. The biochemistry of oxygen - activation and –detoxification analyzed in the past has led to the identification of many similar or more or less identical features in plants and animals. Biochemical model reactions simulating these common situations allow to predict possible functions of plant´s defence molecules and/or systems, to find new fields of application and to exploit up to date unknown resources. In this report potential cooperatively - protecting properties for both animals and plants of certain flavonoids, carotenoids and monoterpenes („essential oils“), based on their biochemical activities in corresponding model reactions, both „in vitro“ and „ex vivo“, are reported. We show that in lipid protection certain substances are only active in the presence of corresponding cooperating partners.

Role of reactive oxygen species, nitric oxide and glutathione in the development of the *Rhizobium* - legume symbiosis

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Reactive oxygen species are generated in the first steps of the *Sinorhizobium meliloti* - alfalfa symbiosis. Superoxide radicals and hydrogen peroxide have been detected in infection threads and there is also evidence for the presence of nitric oxide in young alfalfa nodules. Moreover, rhizobial mutants, with a reduced antioxidant defence, exhibit an impaired capacity to nodulate. The oxidative burst generated in response to symbiotic infection can be consistent with rhizobia being initially perceived as invaders by the plant. However this long-lasting burst is also associated with successful infections. The burst could trigger the expression of plant and/or bacterial genes which are essential for the nodulation process. In this framework, glutathione (GSH) and homoglutathione (hGSH) could be key intermediates for gene expression, via the modification of the redox balance : indeed, transgenic roots with lowered GSH/hGSH levels exhibit an altered nodulation capacity. On the other hand, when nitrogen fixation begins to decline, accumulation of important amounts of hydrogen peroxide is observed in the periphery of the central infected tissue of soybean nodules. Concomitantly, expression of a cysteine protease gene and events related to programmed cell death are observed in this zone; this can be viewed as the onset of the nodule senescence.

Peroxisomes, reactive oxygen species and nitric oxide

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Peroxisomes are cell organelles with an essentially oxidative type of metabolism which carry out a wide variety of important cellular functions [1]. Peroxisomes, like chloroplasts and mitochondria, can generate superoxide radicals ($O_2^{\cdot -}$), and have a complex battery of antioxidative enzymes, apart from catalase, such as SODs, the ascorbate-glutathione cycle, and several NADP-dehydrogenases [2]. In recent years, plant peroxisomes have been shown to have a ROS-mediated metabolic function in plant senescence and abiotic stress situations [1].

The presence of nitric oxide synthase (NOS) was demonstrated in plant peroxisomes and the enzyme was characterized. The peroxisomal NOS was Ca-dependent, constitutively expressed, and immunorelated with mammalian iNOS [3]. Peroxisomes were also found to contain nitric oxide ($NO^{\cdot}$), showing that $NO^{\cdot}$ is an endogenous metabolite of these organelles. Taken together, these evidence suggest that peroxisomes could have a relevant role as a source of signal molecules, like $NO^{\cdot}$, $O_2^{\cdot -}$ and H_2O_2, in the signal transduction pathways of plant cells [4].

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Oxidative stress, antioxidants, and plant mitochondria

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Mitochondria have not traditionally been regarded as a major source of active oxygen species in leaves. As a result of photosynthesis, up to 100 μmol H_2O_2 per mg chlorophyll is produced in the peroxisomes of a C_3 leaf and up to about 30 μmol H_2O_2 in the chloroplasts. In contrast, the maximum rate of H_2O_2 formation due to the mitochondrial electron transport chain is likely to be much less than 1 μmol under the same conditions. Nevertheless, the mitochondrial oxidative load could be crucial in influencing and setting the cellular redox-state. Plants have a robust tolerance of high rates of H_2O_2 formation because of the action of the antioxidative system. Mitochondria, like other compartments of the plant cell, house both enzymic and non-enzymic antioxidants. The last step of ascorbate biosynthesis is located in the inner mitochondrial menbrane, where it is coupled to electron flow. Ascorbate liberated into the intermembrane space is translocated to the rest of the cell. Moreover, mitochondria contain specific soluble and bound forms of ascorbate peroxidase (APX), both in the matrix and the intermembrane space. The distribution of APX between these two mitochondrial compartments appears to be crucial in determining stress resistance. Changes in the redox state of the mitochondrial electron transport chain initiate signalling that resets the antoxidative capacity throughout the cell.

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SESSION VII
LIPOPHILIC ANTIOXIDANTS: VITAMIN E AND COENZYME Q

Action of Vitamin E against Stress on Cells

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The important biological function of vitamin E is, at least in part, to inhibit the free radical-mediated oxidation of biological molecules, which causes deleterious effects. This study was performed to measure and compare the effects of vitamin E homologues, α−, β−, γ−, and δ− tocopherols and tocotrienols, against the cytotoxicity induced by selenium deficiency. It was confirmed that the reactivity toward peroxyl radicals decreased in the order of α > β > γ > δ- forms and that corresponding tocopherols and tocotrienols exert same reactivities. On the other hand, it was also found that tocotrienols moved between the membranes and were incorporated into the cells faster than the corresponding tocopherols. Removal of selenium from the culture medium induced the death of Jurkat cells. Tocopherols and tocotrienols suppressed the cell-death in a dose- and time-dependent manner. Tocotrienols suppressed the cytotoxicity at lower concentration than tocopherols, which was ascribed to a more efficient uptake into cells.

Bioavailability of Vitamin E Forms

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In vitamin E research, 'bioavailability' and 'biopotency' have often been used synonymously creating babbling terminology. For vitamin E, the rat fetal resorption bioassay is the classical test that measures biopotency in terms of preventing embryonic death. However, in humans studies using functional endpoints that reflect potency, such as hemolysis or lipid peroxidation, are limited for tocopherols (natural or synthetic) and tocotrienols.

The Food and Nutrition Board (FNB, 2000) proposed changing the potency ratio of RRR- : all-rac-α-tocopherol for humans from 1.36 to 2.0. However, a critical re-evaluation of all published data in humans that compared plasma responses (bioavailability) to supplemental doses of unlabeled RRR- and *all-rac*-α-tocopherol continues to support the presently accepted potency ratio of 1.36. Recent studies using deuterated tocopherol reveal that the magnitude of ratio changes with dose. While nutritional doses supported the 2:1 ratio, supplemental doses resulted in a ratio close to 1.5. This clearly indicates that no valid conclusion can be drawn from bioavailability studies on the relative potency of *all-rac*- versus RRR-α-tocopherol, even if they employ unlabeled or labeled forms.

Apart from the proposed change in the α-tocopherol ratio, the FNB has suggested that only α-tocopherol bears vitamin E activity. Nevertheless, other vitamin E forms, particularly the most abundant natural form; γ-tocopherol, exerts vitamin E activity too.

Studies applying more meaningful functional endpoints are urgently needed to determine the relative potencies of the various vitamin E forms for humans.

Metabolism of tocopherols and tocotrienols

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The side chains of tocopherols and tocotrienols are degraded by ω- followed by ß-oxidation. The ß-oxidation pathway has been proven by the identification of the final products, carboxyethyl hydroxy-chromans (CEHC) in human urine and plasma, and all possible intermediates in HepG2 cells by GC/MS analysis. The initial ω-hydroxylation is catalyzed by a cytochrome P450 enzyme (CYP). CYP3A4[1,2] and CYP4F2[3] have been identified as candidates from inhibitory, stimulatory and enzymological experiments. CYP4F2 hydroxylated γ-tocopherol with high activity but acted poorly on α-tocopherol[3]. Metabolism of *all rac* α-tocopherol but not γ-tocopherol could be stimulated by rifampicin, an inducer of CYP3A4[2]. CYP3A4 metabolizes about 60% of all drugs nowadays used for therapy. It can be induced by many of its substrates via activation of the pregnane X receptor (PXR). Also α-and γ-tocotrienol induced a PXR-driven CAT reporter in HepG2 cells with an even higher efficacy than rifampicin, whereas δ-, α-, and γ-tocopherol were less active[4]. γ-Tocotrienol as the most active type of vitamin E in activating PXR as well as rifampincin induced endogenous CYP3A4 and CYP3A5 in HepG2 cells about 2-fold. This shows that individual forms of vitamin E are metabolized by and can interact with xenobiotics metabolizing enzymes.

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New findings on the role of vitamin E in skin: *in vitro* and *in vivo* protection against squalene monohydroperoxide generation in solar exposed skin

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At the outermost surface of human skin, skin surface lipids (SSL) are first-line-targets of solar UV radiation. Therefore, we hypothesized that UVA- and UVB-irradiation induce photo-oxidation of SSL. To test this, sebum samples were collected from facial skin of 17 healthy volunteers, weighed and immediately irradiated with either UVB or UVA. Squalene (SQ), the major sebum lipid, as well as photo-oxidation products were identified in sebum lipid extracts by HPLC analysis. Upon UVA exposures squalene and naturally occurring skin vitamin E were dose dependently depleted. Inversely correlated with the depletion of vitamin E and squalene, an unidentified sebum lipid photo-oxidation product (USLPP) strongly accumulated in irradiated sebum. Using HPTLC, HPLC, APCI MS and NMR, USLPP was identified as a mixture of specific squalene monohydroperoxide isomers (SqmOOH). SqmOOH purified from sebum was identical with SqmOOH synthesized by preparative photo-oxidation of SQ. SqmOOH was formed even upon lowest physiological doses of UVA (5 J/cm^2). While physiological baseline levels of SqmOOH in human skin were only slightly above detection limits, SqmOOH levels increased dramatically upon exposure to UVA both *in vitro* and *in vivo*. Based on this principle mechanism, a human sebum photooxidation test (SPT) was developed for screening of photoprotective antioxidants. Alpha-tocopherol, in particular when combined with ascorbic acid, strongly inhibited UVA induced SqmOOH formation *in vitro* (SPT) and *in vivo*. In conclusion, SqmOOH was identified as the most sensitive marker of photooxidative stress to date described for human skin.

Vitamin E prevents foam cell formation in the development of atherosclerosis

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Atherosclerosis, coronary heart disease, heart infarction, and stroke are the leading causes of death in the world. One of the major risk for atherosclerosis is hypercholesterolemia, with elevated levels of cholesterol rich low density lipoprotein (LDL). During atherosclerosis, lipoproteins such as LDL become trapped at the site of lesion and are converted to oxLDL, which contains both oxidized proteins and lipids. Smooth muscle cells become activated by oxLDL, start to proliferate, and migrate into the intima of the arterial wall. OxLDL provokes a cascade of cellular responses at the atherosclerotic lesion, ultimately leading to formation of atherosclerotic plaques. In this process, scavenger receptors could play a critical role because of their ability to bind oxLDL and their function in transporting lipids and cholesterol into and out of the cells.

Some epidemiological studies have shown an association between high intake or high serum concentrations of antioxidant vitamins like vitamin E and lower rates of ischemic heart disease. Vitamin E, inhibits smooth muscle cell proliferation by affecting protein kinase C activity, an effect not related to its antioxidant properties. In our *in vitro* studies, incubation of vascular smooth muscle cells in the presence of 5 μg/ml LDL resulted in stimulation of cell growth and protein kinase C activity. Moreover, vitamin E inhibited LDL induced vascular smooth muscle cell proliferation and protein kinase C activity. On the basis of these *in vitro* results, an *in vivo* study has been carried out. Rabbits were fed with vitamin E poor diet, cholesterol (2 %) supplemented diet, cholesterol (2 %) supplemented diet plus i.m. treatment with vitamin E (50 mg/kg). After 4 weeks aortas were removed and analysed by microscopy for atherosclerotic lesions. Aortic samples were analysed for scavenger receptor expression. They showed typical atheroscle-

rotic lesions in the cholesterol fed group but not in the vitamin E treated one. Aortic smooth muscle cells obtained from cholesterol fed group exhibited an increase of protein kinase C activity and scavenger receptor expression, which was fully sensitive to vitamin E treatment.

Our studies indicated that vitamin E fully prevented cholesterol induced atherosclerotic lesions and foam cell formation by a mechanism involving regulation of protein kinase C activity and down regulation of scavenger receptor expression.

Tocotrienols: their effect on immune modulation and gene expression

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Vitamin E is emerging as an important nutritional supplement not only for its cellular antioxidant and lipid lowering properties, but also as an anti-proliferating agent. It has also been shown to contribute in immunoregulation, antibody production and resistance to implanted tumours. We have shown recently that tocotrienols are the components of vitamin E responsible for growth inhibition in human breast cancer cells *in vitro* as well as *in vivo*, through oestrogen independent mechanisms. Although tocotrienols act on cell proliferation in a dose dependent manner and can induce programmed cell death no specific gene regulation has yet been identified. In order to investigate the molecular basis of the effect of tocotrienols, we performed an array analysis of cancer-related gene expression in oestrogen-dependent (MCF-7) and oestrogen-independent (MDA-MB-231) human breast cancer cell lines. The cells were incubated with 8_g/ml of tococtrienols for 72 hours. In a further experiment, MCF-7 cells were also injected into nude mice and supplemented with 1000 ppm tocotrienols for 20 weeks. At the end of 20 weeks, the tumour tissue was excised and analysed for gene expression. The results indicate that tocotrienols significantly alter the profile of gene expression in all experimental models. Tocotrienol supplementation modulated significantly 46 out of 1200 genes in MDA-MB-231 cells (22 up- and 24 down-regulated). In MCF-7 cells, tocotrienol administration was associated with a lower number of affected genes (3 genes up-regulated and 18 genes down-regulated). Interestingly, only 3 were affected in a similar fashion in both cell lines: c-myc binding protein MM-1, 23-kDa highly basic protein and interferon-inducible protein 9-27 (IFITM-1). These proteins are most likely involved in cell cycle and can exert inhibitory effects on cell growth and differentiation of the tumour cell lines.

The supplementation of tocotrienols in nude mice injected with MCF-7 cells resulted in significant modulation of 30 genes. Among these genes, IFITM-1 was found to be strongly up-regulated, while in both cultured cell lines, we observed a significant down-regulation. The macrophage inhibitory cytokine 1 (MIC1) gene was up-regulated in the tumour of tocotrienol supplemented animals and in the MDA cell line. Finally, tumour from tocotrienol-supplemented animals showed a strong up-regulation of the CD74 antigen which was significantly down-regulated in MDA cells. The most significant data was confirmed by means of either northern hybridization or RT-PCR techniques. These immune-regulatory genes provide evidence as to how the tumours were able to evade host immune response since a tumour did arise in the animal.

These data suggest that tocotrienols are able to affect cell homeostasis possibly independent of their antioxidant activity. This effect is similar between oestrogen dependent and independent cell lines and is observed to be altered after transplantation in living animals.

Cloning of novel human SEC14p-like proteins: Cellular localization, ligand binding, and functional properties

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Cloning and expression of two novel genes highly similar to the tocopherol associated protein (hTAP/SEC14L2/SPF) has been realized. The three recombinant hTAPs have been immunoprecipitated and their associated, lipid soluble molecules were extracted. These proteins bind not only tocopherols, but also phosphatidylinositol, phosphatidylcholine and phosphatidylglycerol. Binding competition studies have shown that phosphatidylcholine, tocopherols and tocopheryl-succinate compete with phosphatidylinositol by associating with hTAPs. Enzymes involved in phospholipids metabolism lend themselves to investigating a possible function of hTAPs. Thus, the activity of recombinant phosphatidylinositol 3-kinase (PI3Kg/p110g) was tested. Recombinant hTAPs were shown to reduce to a half the activity of the recombinant catalytic subunit of phosphatidylinositol 3-kinase gamma. The activity was stimulated in the presence of α-tocopherol up to 5-10 fold. Antibodies against the recombinant hTAP1 were used to immunoprecitate this protein from cells. The immunoprecipitate resulted to contain PI3-kinase activity, indicating a physical contact between the two proteins at a cellular level. In conclusion, hTAPs modulate, in a tocopherol sensitive manner, phosphatidylinositol-3-kinase, a central enzyme in signal transduction, cell proliferation and apoptosis. It is possible that other phosphatidylinositol and phosphatidylcholine dependent signaling pathways are modulated by hTAPs and tocopherols, possibly by transporting and presenting these ligands to the corresponding enzymes.

Mechanism of age and vitamin E induced changes in cyclooxygenase activity

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Accumulating evidence indicates that T cell-mediated immune responses decline with aging. We, as well as others have demonstrated that, in addition to intrinsic changes in T cells, increased macrophage (Mφ)–derived prostaglandin E_2 (PGE_2) production contributes to the age-associated decline in T cell function. We further showed that the age-related increase in lipopolysaccharide (LPS)-induced MØ PGE_2 production is due to increased cyclooxygenase-2 (COX-2) mRNA, and protein levels leading to increased COX enzyme activity. We have shown that the age-associated increase in LPS-stimulated macrophages Mφ prostaglandin E_2 (PGE_2) production is due to ceramide-induced upregulation of cyclooxygenase (COX)-2 transcription that leads to increased COX-2 expression and enzyme activity. To determine the mechanism of the age-related and ceramide-dependent increase in COX-2 transcription, we investigated the role of various transcription factors involved in COX-2 gene expression. The results showed that LPS-initiated activations of both consensus and COX-2 specific NF-κB, but not AP-1 and CREB, were significantly higher in Mφ from old mice than those from young mice. We further showed that the higher NF-κB activation in old Mφ was due to greater IκB degradation in the cytoplasm and p65 translocation to the nucleus. An IκB phosphorylation inhibitor, Bay 11-7082, inhibited NF-κB activation, as well as PGE_2 production, COX activity, COX-2 protein and mRNA expression in both young and old Mφ. Similar results were obtained by blocking NF-κB binding activity using a NF-κB decoy. Further, NF-κB inhibition resulted in significantly greater reduction in PGE_2 production and COX activity in old compared to young Mφ. Addition of ceramide to the young Mφ, in the presence

or absence of LPS, increased NF-κB activation in parallel with PGE_2 production. Bay 11-7082 or NF-κB decoy prevented this ceramide-induced increase in NF-κB binding activity and PGE_2 production. These findings strongly suggest that the age-associated and ceramide-induced increase in COX-2 transcription is mediated through higher NF-kB activation, which is, in turn, due to a greater IκB degradation in old Mφ. Vitamin E has been shown to reduce PGE_2 production while improving the immune response in the aged. In aged mice, we showed that vitamin E-induced decrease in PGE_2 production is due to decreased COX activity. However, vitamin E had no effect on COX mRNA and protein levels, indicating a post-translational regulation of COX by vitamin E. Further experiments indicated that vitamin E decreases COX activity through reducing formation of peroxynitrite, a derivative of nitric oxide shown to be involved in the activation of COX-2. Other homologues of tocopherols were also effective in inhibiting COX activity but their degree of inhibition varied. The varied potency to inhibit COX activity was not explained totally by differences in their antioxidant capacity.

Regulation of the coenzyme Q metabolism

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In order to study the uptake and metabolism of CoQ, labeled form of this lipid was synthesized. CoQ was taken up from the circulation by liver, spleen, adrenals, ovaries, thymus and heart but not into muscle, brain and kidney. Water-soluble metabolites appear in all organs and these can be isolated in urine and feces. The main metabolite was isolated by HPLC procedures and analyzed by mass spectrometry. It was found to consist of an unchanged substituted benzoquinone ring attached to a modified isoprene residue which was phosphorylated. Thus CoQ not only synthesized but also degraded in all cells and tissues, and the main metabolite after derivatization (mainly phosphorylation) excreted into the urine. Two nuclear receptors are involved in the regulation of CoO metabolism. PPARα is necessary for the induction of the lipid synthesis by peroxisomal inducers by not required for the basal synthesis of CoQ and for the lipid increase upon cold exposure. RXRα, on the other hand, is required for the synthesis of this lipid and also for the cold induction, but has no role in the increase upon peroxisomal induction. Dietary CoQ in humans appears in the circulation and taken up by mononuclear but not polynuclear cells. On the other hand, both cell types exhibit increasing vitamin E content. A selective increase of arachidonic acid occurs in phospholipids of the mononuclear cells, indicating an inhibition of phospholipase A_2. The expression of β2-integrin CD11b and complement receptor CD35 on the plasma membrane of monocytes is significantly decreased upon dietary CoQ. CD11b is an important adhesion molecule, eliciting the interaction of monocytes with the endothelial cells, and consequently plays a considerable role in the initiation of atherosclerosis. It appears that the anti-atherogenic effects of CoQ are mediated by other mechanisms beside its antioxidant protection.

SESSION VIII

VITAMIN E AND SELENIUM

Vitamin E in pregnacy and pre-eclampsia

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Pregnancy is associated with marked changes in lipid metabolism and there is a general state of oxidative stress. In women who develop pre-eclampsia this oxidative stress is magnified and indeed, may underlie the development of the condition. Evidence to support this was obtained in an intervention trial in which women at risk of developing pre-eclampsia received vitamin C (1000 mg/day) and vitamin E (400 IU/day) supplements or placebo. (Chappell et al, Lancet 354:810-6, 1999). Increased risk of pre-eclampsia was determined by abnormal uterine artery Doppler waveform analysis or pre-eclampsia in the previous pregnancy. 79 women identified as 'at risk' received vitamin supplements and 81 received placebos. These were compared with 33 low risk women not taking supplements. Blood samples were obtained every 4 weeks from 20 weeks' gestation until delivery. Those receiving vitamins were found to have a markedly reduced incidence of pre-eclampsia. This clinical benefit was associated with improvement in a range of markers of placental insufficiency and oxidative stress. For example, markers of oxidative stress and placental function were abnormal in the placebo group; compared to the low risk women, ascorbic acid concentrations were 28% lower (95% CI 15% to 40%) and 8-epi-$PGF_{2\alpha}$ 49% higher (CI 8 % to 104%). In the vitamin supplemented group, concentrations of ascorbic acid, 8-epi-$PGF_{2\alpha}$, leptin and PAI-1/PAI-2 were not significantly different from the low risk group. These findings are now being followed-up in a number of multi-centre studies to determine if anti-oxidant supplementation of women at risk of pre-eclampsia is associated with improved clinical outcome, supporting a rationale for prophylactic vitamin C and E.

Antioxidant role of selenium: Relevance to health problems

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Selenium, as selenocysteine in selenoproteins, scores over its analogue, sulphur, in existing as an anion (selenolate) at biological pH. This enables it to carry out redox reactions and is the basis of its antioxidant action. A number of selenoproteins have antioxidant functions. The best known example is the family of glutathione peroxidases (GPxs) that are now known to include not only cytosolic, gastrointestinal, plasma and phospholipid hydroperoxide glutathione peroxidases (PHGPx) but also forms similar to PHGPx that have crucial roles in the male reproductive system. Other selenoproteins that appear to have redox functions include thioredoxin reductase, iodothyronine deiodinase, selenoprotein P and prostate epithelial selenoprotein. The antioxidant effects of selenoproteins appear to be relevant in cardiovascular disease, thrombosis, pancreatitis, rheumatoid arthritis, asthma, systemic inflammatory response syndrome (sepsis), viral infection and male and female reproduction. It is therefore of concern that selenium intakes appear inadequate to optimise the activity of the seleno-proteins in some parts of the world. I shall present experimental evidence on an oxidative-stress condition, pre-eclampsia, to show that this is likely to be the case in the UK. However, there are some effects of selenium that appear to require higher dietary intakes than those needed to optimise the activity of the antioxidant selenoproteins viz. immune system and anti-cancer effects. The question is, therefore, whether the antioxidant functions of selenium are relevant to these effects. I shall draw upon evidence from the literature that suggests that although other mechanisms are favoured, the antioxidant properties of the selenoproteins may indeed be important in the anticancer effects of selenium.

Lessons from SelP knock out

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Selenoprotein P (SelP), the major extracellular selenoprotein, was knocked out in mice. The SelP deficient mice showed impaired growth and developed ataxia. Homozygous SelP deficient male mice raised on standard diet were infertile. Se content was elevated in liver but low in plasma and other tissues and selenoenzyme activities (GPx, TrxR) were altered accordingly. In particular the brain, which is not easily depleted by alimentry Se restriction, had low Se levels low GPx and TrxR activities and reduced GPx-1 transcript levels in SelP-deficient mice. Our data disclose that SelP plays a pivotal role in delivering hepatic Se to different target tissues.

Expression of nuclear PHGPx

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Three isoforms of PHGPx mRNAs are transcribed from the single gene *gpx-4*, yielding three proteins differing for their N-terminal extentions. These contain specific nuclear or mitochondrial localization sequences, while absence of extention yields the cytosolic protein. The transcription of the nuclear-type of messenger takes place using an alternative exon recently identified (1). To study the mechanism of transcription, rat *gpx-4* was cloned, 5' UTR of the PHGPx mRNA extracted from different rat cells and tissues analysed by 5' RACE reaction using SMART technology. The transcripts showed distinct 5'UTRs, indicating a mechanism of alternative transcription initiated at alternative promoters. In silico analysis of rat *gpx-4* identified two promoters, one in position equivalent to the previously described human promoter, the other in the area of the alternative exon. In a rat reporter system, the extention of the fragment containing the previously known promoter to the area of the alternative exon encoding for the nuclear sequence, mantained an activity of 50%. Furthermore, in the rat embrional heart line H9C2, promoter activity was not significantly changed when area corresponding to the previously known promoter was cut out. Quantitative evaluation by real time PCR of the nuclear transcript endogenously expressed in the cells used for reporter experiments suggested a wide distribution although in a low amount. A statistically significant higher amount of the nuclear transcript was observed in H9C2 cells. It is therefore proposed the presence of a functional second promoter within *gpx-4*, driving the expression of the nuclear form by alternative transcription. The low measured activity of the novel nuclear promoter is apparently accounted for by the low expression of the nuclear PHGPx transcript in the cells used for transfection experiments.

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SESSION IX

LIPOPHILIC ANTIOXIDANTS: CAROTENOIDS

Carotenoid conversion to Vitamin A: New insights at the molecular level

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Carotenoids are C_{40} isoprenoids synthesized in plants, certain fungi and bacteria with characteristic molecular structures and properties responsible for light absorption as well as for the inactivation of aggressive radicals. Theses compounds are currently intensively studied regarding their potential to lower the risk of chronic diseases. Most important, certain carotenoids are the natural precursors (provitamins) for vitamin A which is essential for vision, cell differentiation, growth control and development.

To become biological active, dietary carotenoids must be first absorbed, then delivered to the site of action in the body and in the case of the provitamin A function, metabolically converted. Despite the general importance of carotenoids in animals, their metabolism has been only marginally characterized since the molecular components involved have remained elusive for a long time. By analyzing blind, rhodopsin-deficient *Drosophila* mutants, we identified the respective genes and characterized their functions including the key enzyme catalyzing carotenoid conversion to vitamin A. Subsequently, we cloned and functionally characterized orthologs of these genes in vertebrates including man. With these tools in hands, we analyzed the impact of this first crucial step in Vitamin A metabolism on animal physiology in more molecular and functional detail.

Effects of β-carotene cleavage products on mitochondrial function

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The studies were undertaken in order to elucidate the role of mitochondria in increasing the risk of cancer after carotenoid intake by individuals exposed to oxidative stress to an unusual extent. We hypothesize that degradation products of β-carotene, which are increasingly formed at heavy oxidative stress, contribute to carcinogenic effects. We investigated whether carotenoid cleavage products (CCP) increase oxidative stress in rat liver mitochondria by impairing mitochondrial function. CCP were obtained by the reaction of β-carotene, retinal, and β-ionone with hypochlorous acid. All CCP strongly inhibited phosphorylating respiration. The presence of 20µM CCP led to a 30-50 % decrease. The mitochondrial GSH dramatically decreased in presence of CCP. In parallel, GSSG concentration increased. Depletion of protein-SH was found after incubation with each of the CCP. MDA levels were enhanced between 7- and 16-fold. It was shown that brain and lung mitochondria were more sensitive towards CCP in comparison with liver mitochondria. The sequence of sensitivity was lung > brain > liver. Based on our data, we identified the adenine nucleotide translocator as that part of oxidative phosphorylation, which was functionally impaired. Elevated accumulation of reactive oxygen species may induce oxidative damage to proteins including the adenine nucleotide translocator, lipids and DNA-molecules in mitochondria and nucleus. Oxidative DNA–damage increases the risk of cancer development. Our data may indicate a basic mechanism of the harmful effects of carotenoids in situations of heavy oxidative stress.

Noninvasive laser Raman detection of carotenoid antioxidants in human skin

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Carotenoid compounds are important ingredients for the antioxidant defense system of the human body and skin. These lipophilic molecules are potent free radical quenchers accumulated in the body through fruit and vegetable consumption. Numerous epidemiological and experimental studies have shown that a higher dietary intake of carotenoids may protect against cancer, age-related macular degeneration, pre-mature skin aging and other pathologies associated with oxidative cell damage. The rapid and non-invasive measurement of carotenoid concentrations in human tissue therefore may be of diagnostic help.

We have used a noninvasive optical technique, based on resonance Raman spectroscopy, to rapidly screen carotenoid levels in human skin and in this way to assess antioxidant status in large populations. Data obtained for a population of 1,375 subjects with a portable Raman scanner revealed that carotenoids are a good indicator of antioxidant status or oxidative stress. The study showed that people with high oxidative stress (for example, smokers and people with high sunlight exposure) generally have low skin carotenoid levels, independent of their dietary carotenoid consumption. We find the Raman technique to be precise, specific, sensitive, and well suitable for clinical as well as field studies. It may become a useful method for the correlation between tissue carotenoid levels and risk for malignancies or other diseases associated with oxidative stress.

Lipophilic micronutrients and sun protection

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ß-Carotene, the most prominent carotenoid, is used for oral sun protection. Based on the antioxidant properties of carotenoids it has been suggested that these compounds exhibit skin protective effects. Carotenoids are quenchers of singlet oxygen and also efficiently scavenge peroxyl radicals.

When ß-carotene was applied as such or in combination with α-tocopherol for 12 weeks, erythema formation (sunburn reaction) induced with a solar light simulator was significantly diminished from week 8 on (1). Similar protective effects are also achieved with a diet rich in lycopene (2). It has been demonstrated that the supplementation with a mixture of carotenoids protects humans against UV-induced erythema as efficient as ß-carotene alone (3). The intake of either ß-carotene (24 mg/d) or a mixture of carotenoids composed of ß-carotene, lycopene and lutein (8mg/d each) led to similar increases in total carotenoids in skin from wk 0 to 12. Long-term supplementation for 12 wk with 24 mg of a carotenoid mix supplying similar amounts of ß-carotene, lutein and lycopene ameliorates UV-induced erythema in humans; the effect is comparable to the treatment with 24 mg of ß-carotene alone.

The extent of protection with ingested carotenoids is not comparable to the use of a sunscreen with a high sun protection factor. However, increasing the basal protection systemically contributes to the permanent defense against UV light-mediated skin damage.

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Carotenoids and cancer prevention

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Carotenoids are likely powerful chemopreventive agents. Regular intake of carotenoids may lower the risk of cancers at different body sites. Recent focus has been on the effects of lycopene, a carotenoid naturally found in tomatoes and tomato-based foods. Epidemiological studies have indicated an association between high blood levels of lycopene and a low risk of prostate cancer. Preliminary findings in patients with prostate cancer indicate that tomato paste or supplements lower tumor progression. The purpose of the presented study was to investigate whether lycopene, alone or in combination with vitamin E, may suppress tumor growth in an animal model of prostate cancer. The human prostate cancer cell line PC-346C was injected in the dorsolateral lobe of athymic nude mice. The animals were fed with synthetic lycopene (5 and 50 mg/kg b.w.), synthetic vitamin E (5 and 50 mg/kg b.w.), a combination of lycopene plus vitamin E (each 5 mg/kg b.w.), or placebo. Tumor growth was determined using transrectal ultrasonography. Plasma was collected for analysis of prostate-specific antigen (PSA). After 91 days of treatment all surviving animals were sacrificed. In mice receiving lycopene plus vitamin E tumor growth was inhibited leading to an increased survival compared to the placebo group (63 days vs. 49 days). PSA levels followed tumor volume in all animals. Overall, the effects of lycopene or vitamin E alone were weaker than their combination. Lycopene and vitamin E may act synergistically in the inhibition of prostate cancer.

SESSION X

FLAVONOIDS, POLYPHENOLS, AND ISOFLAVONES

Cytoprotective and cytotoxic effects of *in vivo* flavonoid metabolites

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In delineating the cytoprotective effects of flavonoids against oxidative stress, few studies have considered the influence of gastrointestinal and hepatic metabolism and the consequences of the ensuing chemical structural changes on their biological properties and activities. Here we present the factors influencing the differential effects of three major classes of flavonoids, the flavonols – represented by quercetin, the flavanols – represented by epicatchin, and the flavanones – represented by hesperetin, and their corresponding in vivo conjugates and metabolites, in protecting human fibroblasts from cell death induced by peroxide-induced oxidative stress. Modifications to flavonoids from aglycone to in vivo forms include increasing polarity and lack of intracellular access through glucuronidation, on the one hand, and decreasing polarity and as well as modulation of redox properties through methylation of catechol moieties, on the other hand.

The results show that epicatechin protects against apoptosis induced by oxidative stress through mechanisms involving the modulation of JNK-activation. Interestingly, its methylated forms (with substituted catechol structures) are equally efficacious suggesting the lack of requirement for the redox-active catechol moiety in its mechanism of action. The glucuronide, however, is ineffective, suggesting a requirement for intracellular access or that the A-ring, modified on glucuronidation, might mediate specific binding.

In contrast, hesperetin (as well as its glucuronides) are neither cytoprotective nor cyotoxic at the concentrations studied. In fact, hesperetin is handled rather differently in that intracelluar metabolism leads to glucuronidation and subsequent export from the cells.

The cellular interactions of quercetin (the most redox-active of flavonoid structures) and its methylated metabolites yet again contrast with those of the flavanol and flavanone and are dependent on the precise structural chemistry of the metabolites and their abilities

to participate in intracellular oxidative metabolism, resulting in cytoxicity or cytoprotection. The question as to whether intracellular glutathionyl conjugate formation is the cytotoxic agent or whether it is merely functioning as the means to detoxification will be discussed.

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Flavan-3-ols and procyanidins: beyond their free radical scavenging properties

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The regular consumption of flavonoid-rich foods has been associated to positive health effects, particularly with regard to vascular physiology. These effects are ussualy explained by the free radical scavenging properties of flavonoids. However, considering blood plasma concentrations, flavonoids could hardly compete with other antioxidants (ascorbate, tocopherols, etc.). We studied some alternative mechanisms that could explain the health effects of flavonoids, assaying cocoa flavan-3-ols and related procyanidins in different in vitro systems. From the obtained results we conclude that: i) flavan-3-ols and procyanidins interact with lipid membranes. This interaction depends on lipid composition, flavonoid structure and procyanidin degree of oligomerization, and was achieved at micromolar concentrations; ii) flavan-3-ols and procyanidins inhibit competitively angiotensin converting enzyme (ACE) activity at physiologically relevant concentrations. This inhibition was maximal for the hexameric procyanidin; iii) flavan-3-ols and procyanidins inhibit DNA oxidation and regulate gene expression in cultured cells. Considering these results, we propose that the interaction of flavonoids with lipids, proteins and DNA, could define flavonoids actual concentrations and functions.

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Scanvenging effects of pycnogenol on free radicals generated from cigarette smoke and detoxification effects

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It is well known that smoking is harmful for human health. It has been found by epidemiological studies that smoking causes high mortality by initiating heart diseases and cancer. The incidence of heart diseases is proportional to the number of cigarettes smoked and about 30% of lung cancer is caused by cigarette smoking. If a material can be found to scavenge efficiently the free radicals in the gas phase of cigarette smoke and to reduce the toxicity of cigarette smoke for humans, it will protect human health. Pycnogenol isolated from the bark of the French maritime pine has very strong antioxidant and effectively scavenging effect on free radicals. In order to study the scavenging effect of Pycnogenol on free radicals generated in the gas phase from cigarette smoke, we developed a spin trapping method to detect alkyl and alkoxyl free radicals in the gas phase generated from cigarette smoke. It was found that Pycnogenol contained in the filter of cigarettes at 1.2 mg, 2.7 mg and 4.0 mg could scavenge the free radicals at about 23%, 27% and 29%, respectively. Ames tests showed that the mutagenic effect of the condensate from cigarette smoke was significantly reduced by Pycnogenol-containing cigarette filters compared to control (the inhibition rate of 1.2mg and 4.0mg pycnogenol were 35.4% and 39.5% respectively). Animal experiments showed that the acute toxicity of cigarette smoke with Pycnogenol in the filter was also significantly reduced in comparison with control cigarettes (the inhibition rate of 1.2mg and 4.0mg pycnogenol were 47.5% and 70.5% respectively). Biopsy and histopathological examination also showed that pycnogenol in the filter could protect the animals from the damage of smoking. Micronuclear experiment showed that Pycnogenol could inhibit the mutagenic effect of cigarette smoke for animals.

Effect of genistein and daidzein on platelet aggregation and monocyte and endothelial function

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There has been much recent interest in the cardiovascular benefits of dietary isoflavones. The aim of the present *in vitro* studies was to investigate potential antithrombogenic and antiatherogenic effects of the isoflavones genistein and daidzein in platelets, macrophages and endothelial cells. Pretreatment with either isoflavone inhibited collagen-induced platelet aggregation in a dose-dependent manner. In a macrophage cell line (RAW 264.7) activated with interferon gamma plus lipopolysaccharide, both isoflavones were found to inhibit nitric oxide production and tumor necrosis factor alpha (TNF-α) secretion dose-dependently. Both isoflavones also dose-dependently decreased monocyte chemoattractant protein-1 secretion induced by TNF-α in human umbilical vein endothelial cells (HUVEC). Compared with daidzein, genistein exerted greater inhibitory effects for all parameters examined. We are currently studying the effect of isoflavone gut (equol, o-desmethylangolensin and liver metabolites (e.g. genistein and daidzein sulfates) on platelet aggregation and monocyte and endothelial function.

cDNA arrays as a tool to investigate the role of nutritionally-relevant molecules in human health and disease

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The determination of the global picture of the effect of nutrients on gene expression through genomic techniques, is fundamental for a better understanding of their activity at the molecular level. cDNA microarrays in combination with proteomics represent new avenues in understanding gene regulation and signal-transduction pathways. DNA arrays are based on the hybridization of labelled cDNA, obtained from the reverse transcription of RNA extracted from cells or tissues with a large number of specific genomic DNA fragments spotted and immobilized onto a nylon membrane, plastic or glass. The intensity of the hybridization is proportional to the level of expression of specific mRNA. As a result differential expression of specific genes, either in response to a stimulus or in the presence of a pathological condition can be assessed. The search for differentially expressed genes provides a better insights into the molecular mechanisms of nutrients enabling the design of related “hypothesis driven” experiments.

In this report our recent results based on cDNA array technique and concerning the effect of molecules of nutritional interest on cellular response to different stimuli will be presented and discussed. They are: i) the effects of a-tocopherol and catechin on the response of primary human endothelial cells challenged with oxidized LDL; ii) the effect of luteoflavanols on the response of the human tumour line U937 to oxidative damage; iii) the effect of tocotrienols on gene expression in both oestrogen dependent and independent human breast cancer cell lines; iv) the differential effect of the procyanidin-rich extract from the maritime pine bark on normal and cancer human cells.

The antiatherogenic and anti-inflammatory potential of oat phenolics

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Avenanthramides are the main polyphenolic antioxidants in oat grains. Avenanthramide A, B, and C are the major constituents of the total antioxidant polyphenolics in oats. We tested the potential antiatherogenic and anti-inflammatory activity of purified oat extract (OExt) by examining its effect on adhesion of monocytes to human aortic endothelial cell (HAEC) monolayers, expression of adhesion molecules, and production of proinflammatory cytokine and chemokines by HAEC. The OExt was prepared and purified by column chromatography. The OExt had no toxicity to HAEC as tested up to 40 μg/mL. The pre-incubation of HAEC with 4, 20, and 40 μg/mL of OExt for 24 h significantly decreased adhesion of U937 monocytic cells to IL-1β-stimulated HAEC in a dose-dependent manner. The effect of 40 μg/mL was comparable to the effect observed with 17 μg/mL of vitamin E. Pre-incubation of HAEC with OExt at 20 and 40 μg/mL, but not at 4 μg/mL, for 24 h significantly suppressed IL-1β-stimulated expression of adhesion molecules ICAM-1, VCAM-1, and E-selectin, and secretion of proinflammatory cytokines IL-6, and chemokines IL-8 and MCP-1. Our data provides first time evidence for potential antiatherogenic and anti-inflammatory effects of polyphenolic antioxidants present in oat grain, which is in addition to its known health benefit effect through its soluble fiber content.

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Wine antioxidants and LDL protein folding

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A rationale for a protective role of wine against atherosclerosis is presented. The following experimental evidence supports the suggested mechanism.

1. The plasma level of lipid hydroperoxides increases in the post-prandial phase.
2. This increases is lower when the same food food is taken with either red wine or grape seed procyanidins.
3. Post prandial LDL is more susceptible to lipid peroxidation than fasting LDL and the increase is prevented if wine is taken with foods.
4. Minimally oxidized LDL- increases in the post prandial phase.
5. LDL- drives the oxidation of whole LDL fraction.
6. In LDL- apoB secondary structure is deeply altered (misfolding and prevalence of β *vs* α structure).
7. Alteration of water-lipid boundary in LDL, produced by lipid hydroperoxides or lysophospholipids accounts for conformational shift of apoB secondary structure.
8. Consistently, stabilization of secondary structure of apoB (e.g. by estradiol and possibly other related polyphenols) plays a major antioxidant effect.

In conclusion, wine procyanidins, apparently active as chain breaking antioxidants during gastric digestion, prevent the post prandial increase of plasma lipid hydroperoxides, in turn perturbing the structure of LDL and thus leading *in vivo* to a lipoprotein containing misfolded apoB. This LDL reproduces all the biological effects of atherogenic minimally modified LDL produced *in vitro*.

Transcription factor switching by curcumin

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Curcumin is a major and the most biologically active component of tumeric, which is consumed daily by 25% of the world's population. Although used in Eastern medicine for centuries, in the West, recent attention has focused on its purported anti-inflammatory, tumoricidal, and chemopreventative properties. The mechanism(s) through which it protects against oxidative injury are unclear.

As with other electrophilic compounds, there is the chemical potential for reaction with oxidants; however, at concentrations that would be reached physiologically, it is more likely that curcumin acts through effects on cell signaling, a property of many electrophiles. Indeed, curcumin exposure increases cellular glutathione (GSH) content through an increase in transcription of both subunits of glutamate-cysteine ligase (GCL), a property it shares with other electrophiles. It also increased mRNA of other Phase II enzymes, NQO1 and GSTA4-4, as well.

In examining the mechanism for the induction, we measured binding of transcription factors to DNA sequences identical to putative response elements in the *gcl* promoters. Curcumin caused modest but sustained increases in binding of proteins to DNA sequences for both TRE and EpRE *cis* elements. More noteworthy however, the compositions of the binding complexes and nuclear content of these proteins was markedly altered. JunD and c-Jun content increased in AP-1 complexes, and JunD increased while MafG/MafK decreased in EpRE complexes. Reconfiguration of AP-1 and EpRE transcription factor complexes likely accounts for increased gene expression of GCL and other Phase II enzymes by curcumin.

Mechanism of Action of Flavonoids: *in vitro* to *in vivo*

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Flavonoids as dietary constituents have attracted much recent interest. Research ranges from chemical and biochemical reaction pathways to biology, nutrition and medicine. We have addressed the topic of cocoa procyanidins as possible items of interest in studies on endothelial dysfunction, related to cardiovascular risk. Biochemically, various lipoxygenases were examined as to their sensitivity toward flavan-3-ols (1,2), and the results suggest that at levels which may occur *in vivo* there is a modulation of lipoxygenases interpretable as beneficial in vascular biology.

In human volunteers, the *in vivo* response to cocoa procyanidins was examined. Measuring the time-course of flow-mediated dilation of the brachial artery as well as the plasma levels of S-nitrosothiols and N-nitrosothiols (3,4), our preliminary results reveal direct effects beneficial to vascular systems compromised in individuals afflicted by one or more of the established cardiovascular risk factors.

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POSTERS

Antioxidant activity of merbau crude extract

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Merbau crude extract (Intsia Palembanica, Leguminosae), 700 mg/kg injected intraperitoneally (i.p.) in rats elicited a reduction in the levels of liver malondialdehyde (measured as TBARS, thiobarbituric acid reactive substances) when compared to controls without affecting the other organs. Lower doses of merbau did not alter TBARS levels in all rat organs. In mice, merbau (200-700 mg/kg) significantly reduced liver and lung TBARS as compared to controls even though the relative organ weights of the liver, lungs and kidney were increased by merbau (100-400 mg/kg). Plasma levels of aspartate transaminase (AST) and alanine transaminase (ALT) were significantly reduced in mice by merbau (50-400 mg/kg). In the DPPH assay, merbau showed potent radical scavenging effects, comparable to those of purified polyphenols i.e. quercetin, catechin and myricetin while alpha tocopherol was without effect. Partially purified merbau faction (WB19d) also scavenged DPPH radical at a comparable rate as the crude extract. However, radical scavenging effects of both the crude extract and WB19d were smaller that that of TROLOX. In microsomal systems undergoing tert-butyl hydroperoxide induced lipid peroxidation, merbau elicited an IC_{50} value for inhibition of lipid peroxidation that was comparable to the IC_{50} value of alpha tocopherol acetate. WB19d elicited an IC_{50} value that is half that of the crude extract while the IC_{50} value for TROLOX was twice the value for alpha tocopherol acetate.

Thus, rats and mice receiving non lethal doses of merbau produced less lipid peroxidation products *in vivo* as compared to con-

trols. Their plasma levels of liver marker enzymes were lower, they also appeared healthier. Crude extract of merbau and WB19d showed *in vitro* antioxidant and free radical scavenging activities that were similar, if not superior to those of alpha tocopherol acetate or TROLOX.

Cytoprotection against oxidant agents by metallothioneins

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Metallothioneins (MT) are low molecular weight proteins involved in homeostasis of physiological cations and in detoxication of xenobiotic metals. Their abundance in thiol groups, from their cysteine residues, provides a remarkable functionality in maintaining the cellular redox balance. MT encapsulated by hypotonic dialysis into carrier red blood cells (RBCs) protect these cells against oxidative action of chemicals (ascorbate/Fe^{3+}), while keeping their haemoglobin functional. This opens the possibility of applying MT-loaded RBCs as protectors towards oxidative stress or as potential detoxication agents.

Macrophages (MΦs) are the most active phagocytic cells in the immune system, maintaining defense mechanisms mainly through formation of reactive oxygen (ROS) and nitrogen (RNS) species. Production of NO by peritoneal MΦs stimulated with LPS and/or IFNγ is very high and reaches maximal concentration in the medium 20 h after application of such iNOS inductors. The presence of MT in the culture reduces the spectrophotometrically detected nitrite concentration in the medium, consequently iNOS activity was determined through radioactive citrulline formed from [^{3}H]-arginine as substrate. Results reveal that the presence of MT in the culture does not affect iNOS activity of peritoneal MΦs, while it does reduce nitrite concentration in the medium in a dose-dependent manner, so exerting a buffering action against excess NO produced. Under the two conditions studied, MT act as scavengers for reactive species, confirming their effective cytoprotective role against oxidants, which under certain conditions, could originate a severe oxidative stress inducing cellular apoptosis.

The reduction of Coenzyme Q_9 at the plasma membrane of *Caenorhabditis elegans* provides antioxidant protection against lipid peroxidation that can not be afforded by Demethoxy-Q_9

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The incubation of plasma membrane purified from wild type *Caenorhabditis elegans* (N2) with NADH had as a consequence the reduction of the endogenous coenzyme Q (Q_9). When these worms were fed with the wild type *Escherichia coli* (OP50) reduction of the exogenous Q_8 was also observed in plasma membrane from *C. elegans*. Although in a very small amount, plasma membrane from wild type *C. elegans* fed with OP50 also contains an intermediate of the biosynthetic pathway of Q_9, the 2-nonaprenyl-3-methyl-6-methoxy-1,4-benzoquinone (demethoxyquinone-9, DMQ_9). This DMQ_9 was not detected, however, when N2 worms were fed with a Q_8-deficient *E. coli* strain (GD1), having, as a counterpart, an increased amount of Q_9 in its plasma membrane.

Long-lived *C. elegans* mutants, *clk-1*, have a deficient Coq7p protein, which is involved in the biosynthesis of Q. As a result, none or very little amount of Q_9 is synthesized in these mutants, and they accumulate the intermediate DMQ_9. Although it has been proposed that DMQ_9 can partially drive the mitochondrial electron transport chain (Felkai *et al.*, EMBO J., 18:1783-1792, 1999), DMQ_9 can not fully replace the biological functions of Q_9 as worms population die unless an exogenous source of Q is administered with the diet. When plasma membrane from a *clk-1* mutant, *qm51*, was incubated with NADH no reduction of DMQ_9 was observed. As previously observed with N2 worms, the small amount of Q_9 present in *qm51* worms was also reduced upon incubation with NADH.

A primary outcome of the difference for reduction between Q_9 and DMQ_9 was the antioxidant capacity of these two molecules. Plasma membrane from *qm51* worms was more sensitive to lipid peroxidation, induced by the azo-initiator AAPH, than those from

N2 worms. Our results indicate that DMQ_9 can not replace Q_9 as an antioxidant in the plasma membrane of *C. elegans*. These results could in part justify the necessity of Q in places other than mitochondria.

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Comparison of antioxidant activities of anthocyanins against LDL oxidation promoted by peroxyl radicals and ferrylmyoglobin

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Red wine polyphenols, including anthocyanins, have been suggested to be greatly responsible for the beneficial effects of regular consumption of red wine against atherosclerosis, mainly through protection of low-density lipoproteins (LDL) to oxidative damage. The aim of this study was to evaluate and to compare the activities of four anthocyanins (cyanidin, malvidin, malvidin-3-glucoside and pelargonidin) against LDL oxidation promoted either by the azocompound 2,2´-azobis-(2-amidinopropane) (AAPH) or ferrylmyoglobin, a relevant physiological oxidant. The extent of LDL modification was evaluated by the fluorescence decay of incorporated *cis*-parinaric acid (PnA) and by the conjugated dienes formation. All the anthocyanins afforded a significantly higher concentration-dependent protection to PnA oxidation, induced by both oxidants, over to ascorbate. Also, they increased significantly the lag time of conjugated dienes formation, as compared with either Trolox or ascorbate. Our data indicate that although all those anthocyanins are potent antioxidants, protecting efficiently peroxyl radical- or ferrylmyoglobin-induced oxidation, their antioxidant activities are highly modulated by the substitution patterns in the B-ring and glycosylation at C-3 position. Thus, consumption of anthocyanins through the intake of red wine may greatly contribute to protect LDL from oxidative damage and therefore, may be relevant in the context of atherosclerosis protection.

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The effect of palm vitamin E supplementation on certain trace elements and antioxidant enzymes in chronic hepatitis B carriers

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Patients with liver diseases have been found to have low levels of scavenger enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) while the production of reactive oxygen species increases. The activities of these enzymes depend on trace elements, which are essential constituents of cell molecules. Vitamin E has been shown to have a protective function against oxidation related disease and liver damage. To determine the effect of palm vitamin E (palmvitee) on trace elements and antioxidant enzymes, a randomized placebo-controlled trial was conducted on chronic hepatitis B carriers. Subjects were given a 200 mg capsule of palmvitee or placebo for a 24-week period. Blood was taken at 0, 6, 12 and 24 weeks, respectively. Plasma vitamin C and E levels were measured using HPLC. Initial observation showed no differences in SOD and GSH-Px activities between normal controls and hepatitis B carriers whilst CAT were higher in hepatitis B carriers. These parameters remained unaffected by palmvitee supplementation. While vitamin C levels remain unchanged, plasma vitamin E levels increased ($p < 0.05$) in the supplemented group beginning 6 weeks after supplementation. Trace elements such as cobalt (Co) and manganese (Mn) were significantly higher while chromium (Cr) and magnesium (Mg) were lower in hepatitis B carriers. Liver function test were within normal limits but alanine aminotransferase decreased (24.3 ± 21.4 UL to 18.0 ± 16.0 U/L) in hepatitis B carriers with palmvitee supplementation. In conclusion, CAT activity, Co and

Mn was higher in hepatitis B carriers compared to normal, while Cr and Mg were lower. Palmvitee supplementation did not affect these values. However, some hepatoprotective effect was observed with supplementation as seen from the reduction in ALT activity.

NADP(H):Quinone oxidoreductase 1 and the regulation of reactive oxygen species levels in animal cells

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NAD(P)H:quinone oxidoreductase 1 (NQO1, formerly known as DT-diaphorase, EC 1.6.99.2) is a cytosolic flavoenzyme ubiquitously present in all the tissues of nearly all animal species. This enzyme catalyzes the two-electron reduction of several quinone substrates and has been recognized as an important member of the antioxidant machinery in the cell (Jaiswal, 2000, *Free Radic. Biol. Med.* **29,** 254). We have investigated the participation of NQO1 in the regulation of reactive oxygen species (ROS) levels in different cellular models, and have tested the role of this enzyme in the control of cell growth and death. In HeLa cells, a cellular model in which cell density is an important factor that regulates growth, NQO1 levels were significantly higher in dense compared to sparse cultures. Both peroxide and superoxide were decreased in HeLa cells at high density. In HL-60 cells, ROS increased transiently after serum removal, but then they tended to decay and recover basal levels. Inhibition of NQO1 with dicumarol in the absence of serum had no effect on peroxide levels, but inhibited the late decrease of superoxide. Oxidative stress caused by NQO1 inhibition in the absence of serum profoundly affected progression of HL-60 cells through the cell cycle and activated cell death.

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Increased superoxide generation is associated with the development of pulmonary hypertension in fetal lambs: A role for NADPH oxidase

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Ligation of the ductus arteriosus in utero produces pulmonary hypertension and vascular remodeling in fetal and newborn lambs. However, the mechanisms producing these vascular changes are not well defined. Because reactive oxygen species (ROS) have been implicated as mediators of smooth muscle cell proliferation, we hypothesized that increased formation of ROS may be involved in the pathophysiology of pulmonary hypertension after in utero ductal ligation. Using ethidium fluorescence we demonstrated an increase in superoxide levels within 2-days of ductal ligation compared to control lungs ($P < 0.05$) localized to the adventitia and smooth muscle cells of hypertensive vessels. SOD-1 and SOD-2 protein levels and activities in lung, vein and artery of hypertensive lambs were unchanged relative to controls at 2-days. However, after 9-days, SOD activity was significantly decreased in arteries from ligated lambs without associated changes in SOD protein expression ($P < 0.05$). Examination of NADPH oxidase expression as a potential source of the superoxide production indicated that the levels of $p67^{phox}$, a subunit of the NADPH oxidase complex, were significantly increased in the pulmonary arteries, but not veins, from the ligated lung as early as 2-days ($P < 0.05$). Functional analyses demonstrated that reducing superoxide levels significantly increased the NO-mediated relaxation of isolated pulmonary arteries after 9-days, but not 2-days, of ductal ligation ($P < 0.05$). These results suggest that increased NADPH oxidase mediated increases in superoxide levels in the PPHN lung leads to the development of endothelial dysfunction.

Phytoestrogens from soy protect against oxidative stress by inducing antioxidant enzyme expression

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Soy beans contain phytoestrogens, which are plant-derived estrogens, structurally similar to 17β-estradiol. Due to their similar chemical structures to 17 β -estradiol, phytoestrogens have antioxidant properties. Genistein, the most abundant phytoestrogen in soy beans, has been shown to increase the activity of antioxidant enzymes such as catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase.

Using adult rats fed a soy or soy free-diet, we determined differences in oxidative stress by measuring levels of an endogenous antioxidant in cells, such as reduced glutathione (GSH), and a lipid peroxidation marker such as malondialdehide (MDA) in isolated mitochondria from the liver of these rats. We also investigated the effect of the soy diet on the expression of mitochondrial antioxidant enzyme manganese-superoxide dismutase (Mn-SOD) by RT-PCR in liver from these rats.

Our results showed that mitochondrial GSH levels were significantly higher in liver from soy fed rats than in those fed a soy free-diet. Regarding lipid peroxidation, we found that mitochondrial MDA levels were lower in liver from soy fed rats than in soy free fed rats. Thus, oxidative stress was lower in soy fed rats than in to rats fed with a soy free diet. We previously found that estrogens stimulated the expression of Mn-SOD. We now report that phytoestrogens also induce the expression of this enzyme.

We conclude that dietary phytoestrogens from soy can protect cells against oxidative stress by inducing the expression of antioxidant enzymes, and thus protecting the cardiovascular system against atherosclerosis and other cardiovascular diseases.

Kidney Mitochondrial Nitric Oxide Synthase

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Nitric oxide synthase (mtNOS) activity was recognized in rat renal cortex mitochondria with NO production rates of 0.14-0.78 nmol/(min. mg protein). The K_MO_2 for kidney mtNOS was determined as 37 μM. Rat pretreatment with enalapril (30 mg/(kg. day) i.p., up to 15 days) increased NO production in kidney, liver and heart mitochondria. In kidney, mtNOS activity and mtNOS protein (measured by Western blot densitometry) were 5 and 2.3 times increased, respectively, by enalapril treatment. EPR analysis with the probe MGD-Fe detected NO production in mitochondria isolated from enalapril-treated rats, but not in control untreated animals. Polyclonal antibodies anti-iNOS and anti-nNOS detected kidney mtNOS in Western blots and inhibited mtNOS biochemical activity. The enzymatic activity of kidney mtNOS generates intramitochondrial NO concentrations that regulate mitochondrial functions: state 3 respiration was decreased by 12 28%; and state 4 H_2O_2 production was increased 12-35% by mtNOS functional activity (the difference between arginine and L-NMMA supplementation) .The competitive inhibition of cytochrome oxidase by NO depends on the simultaneous O_2 concentration. At the physiological level of 20 μM O_2, NO steady state concentration is 39 nM, with an O_2/NO ratio of 513 and a cytochrome oxidase inhibition of 26 %. The upregulation of mtNOS is one of the current hypothesis for the beneficial effects of the inhibitors of the renin-angiotensin system upon aging. The production of NO by mtNOS appears as a regulatory process that modulates kidney mitochondrial oxygen uptake and cellular energy production.

The effects of tocopherols on the Ca^{2+}-response under xanthine oxidase-catalyzed ROS and thapsigargin-induced $[Ca^{2+}]_i$-oscillations in endothelial cells

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The major area of interest concerning vitamin E lies essentially in its antioxidative capacity. In our study we investigated xanthine oxidase (XO)-catalyzed reactive oxygen species (ROS) on human microvascular endothelial cells (HMEC-1) and the influence of α-, β- or γ-tocopherol on XO-catalyzed ROS. In dependence of XO concentration ROS generation caused varying increases in cytosolic Ca^{2+} ($[Ca^{2+}]_i$) levels. XO showed a dose dependent increase in $[Ca^{2+}]_i$. Dependent on the XO concentration tocopherol was able to lower the oxidative stress induced increases in ($[Ca^{2+}]_i$).

On thapsigargin (TG) stimulated $[Ca^{2+}]_i$-oscillations in HMEC-1 ROS generation resulted in considerable variations. Preincubation with physiological concentrations of any of the different tocopherols had no influence on this stress response. However, the frequency of the TG-induced oscillations was changed in both directions in comparison to control cells due to the respective tocopherol applied. The regulation of the frequency of ($[Ca^{2+}]_i$) oscillations may provide cells with a specific mechanism to control signalling and may have advantages in controlling cellular responses.

Thapsigargin induced apotosis ocurred with mitochondrial and reticulum luminal alterations associated with NO production

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The purpose of this study was to analyse the molecular mechanisms associated with rat thymocytes apoptosis induced by thapsigargin, an inhibitor of endoplasmic reticulum Ca^{++}-ATPase pump. In these cells as well as in other cellular systems cytosolic Ca^{++} increase after thapsigargin treatment is a well known event. Our results showed that apoptosis induced by thapsigargin was associated with a marked and sustained mitochondrial depolarization after 3 and 6 h of drug exposure, measured with the potentiometric probes DiOC6 and JC-1 respectively. These mitochondrial changes were associated with alterations of the luminal endoplasmic reticulum protein glucosiltransferase (UDP-GT), involved with the quality control process during protein folding. An increase in glucosiltransferase activity and glucosiltransferase mRNA expression were observed, as compared with untreated cells, after 1.5 hours of 500 nM thapsigargin treatment. This fact was associated with a significant increase in nitric oxide production by endoplasmic reticulum in isolated membranes from treated thymocytes, being the L-NMMA sensitive NO generation 0.35 nmol/min per mg protein and 1.9 nmol/min per mg protein in untreated and thapsigargin treated thymocytes respectively. This indicates the presence of a nitric oxide synthase associated to the endoplasmic reticulum, activated by cytosolic Ca^{++} increase after thapsigargin exposure. Simultaneously and as a consecuence of the endoplasmic reticulum stress response the protein UDP-GT, important contributor to the correct protein folding, is activated. We can conclude that Ca^{++} as well as NO are important mediators during thapsigargin induced apoptosis, being able to induce signalling pathways connecting endoplasmic reticulum and mitochondria, which results in coordinate alterations of cellular structures and functions during the execution of the death program.

Tamoxifen prevents the Mitochondrial Permeability Transition induced by Pro-oxidants

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Tamoxifen (TAM), the widely prescribed anticancer drug in the therapy and prophylaxis of breast cancer, inhibits the mitochondrial permeability transition (MPT) induced by Ca^{2+} plus inorganic phosphate. Increasing evidences have identified mitochondria as a strategic centre that determines cell's demise, since they are the generators of the cell energy as well as a target on the process of cell injury induced by xenobiotics. In particular, the MPT has been widely implicated in the regulation of assorted cell functions, in the mechanisms of chemical-induced tissue injury and apoptosis. Pro-oxidants, such as peroxynitrite and *tert*-butylhydroperoxide, induce oxidation of membrane protein sulfhydryl groups, matrix GSH and pyridine nucleotides [(NAD(P)H], closely followed by an increase in the Ca^{2+} concentration and generation of ROS within the mitochondria, leading to the MPT induction. Accordingly, antioxidants are expected to prevent the induction of this process involved in the apoptosis activation. TAM has been shown as an efficient inhibitor of lipid peroxidation and of the MPT. Therefore, the aim of the present work was to identify mechanisms of MPT inhibition by TAM in order to further characterize its mechanisms of citotoxicity. The MPT was induced by pre-incubating mitochondria with pro-oxidants and Ca^{2+} and was characterized by an extensive swelling, depolarization of membrane potential ($\Delta\Psi$), Ca^{2+} release and NAD(P)H degradation. TAM that mimics cyclosporine A in inhibiting the MPT, also prevent the mitochondrial swelling, depolarization of $\Delta\Psi$, Ca^{2+} release and the NAD(P)H oxidation, suggesting that the MPT inhibition by TAM may be related to its antioxidant activities.

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Amyloid-β and H_2O_2 induced impairment of the glutathione cycle: Involvement of oxidative stress

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Oxidative stress has been implicated in Alzheimer's disease due, in part, to the action of amyloid β-peptide (Aβ). We observed that Aβ 25-35 induced an increase in reactive oxygen species (ROS) in NT2 ρ^+ cells, leading to protein and lipid oxidation. This oxidative status was partially prevented by the antioxidants, vitamin E, reduced glutathione, and by melatonin. Upon Aβ 25-35 treatment, in ρ^+ cells, a decrease in glutathione reductase activity and in GSH levels was observed, whereas glutathione peroxidase activity was shown to be increased. However, NT2 ρ^0 cells (that lack mitochondrial DNA), in the absence of Aβ, showed an increase in ROS production, lipid and protein oxidation, as compared with parental ρ^+ cells. In NT2 ρ^0 cells, in the absence of Aβ, GSH levels were maintained, whereas glutathione reductase and peroxidase activities were increased. The exposure of ρ^0 cells to Aβ did not induce any significant change in these parameters. Because Aβ-induced cell toxicity may occur independently of generation of ROS, we have also analysed the regulation of enzymes of the glutathione cycle in ρ0 and ρ+ exposed to H_2O_2. H_2O_2 induced an over-stimulation of glutathione reductase activity. An improved antioxidant status in ρ0 cells was shown to explain a decreased formation of intracellular hydroperoxides and protein carbonyl groups induced by a brief exposure to H_2O_2. Considering the evidences presented, we argue that Aβ-induced toxicity is mitochondria specific and involves other aspects than oxidative stress.

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Mitochondrial nitric oxide synthase and redox signaling during rat liver development

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In the last years, different mitochondrial nitric oxide synthase isoforms have been described in rat and mouse tissues such as liver, skeletal muscle and, more recently, heart and brain. The regulation of mtNOS results in the modulation of oxygen uptake and reactive oxygen species production, which in turn may regulate the activity of cell cycle regulatory proteins determining proliferation or cell cycle arrest. The aim of this study was to evaluate the modulation of liver mtNOS and the resultant hydrogen peroxide steady-state concentration during rat liver development and a possible relation to proliferative and quiescent cell stages. In this study, the embryonic days 17 and 19 of gestation and postnatal day 2 represent proliferative hepatocyte phenotype and postnatal 15 to 90 the quiescent phenotype. MtNOS was almost undetectable in fetal liver and progressively increased after birth up to adult levels at P30. NO-dependent mitochondrial H_2O_2 production and Mn-SOD paralleled the developmental modulation of mtNOS activity. Proliferative hepatocyte phenotypes showed higher phospho-ERK1/2 and cyclin D1 expression and lower phospho-p38 MAPK expression while quiescent phenotypes showed an opposite pattern. Treatment of P2 isolated hepatocytes with NO and H_2O_2 level modulators resulted in changes in [3H] thymidine incorporation. The present results suggest that modulation of NO and H_2O_2 steady-state concentrations during liver development contributes to regulate MAP kinase activities and cyclin D1 expression leading to proliferation or cell cycle arrest.

Vitamin E recommended daily intake is not enough to avoid the oxidative stress induced by intense exercise

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Intense physical exercise has been associated with an increase of free radical production and a direct relationship with lipid peroxidation has been reported. Some studies suggested favourable effects of antioxidant vitamin supplementation on lipid peroxidation after exercise whereas others failed to demonstrate these effects. Far fewer studies have examined the plasma carotenoid pattern of change in response to a physical activity. The purpose of this study was to analyse the changes in plasma lipophilic antioxidants and oxidative stress markers in professional cyclists during a mountain stage. Cyclists vitamin E ingestions were in the range of the recommended daily intake. We determined plasma carotenoids and plasma and lymphocyte vitamin E concentration before and three hours after exercise. We also determined two oxidative stress biomarkers: malondialdehyde (MDA) in plasma and carbonyl concentration in plasma and lymphocytes. We observed a differential pattern of change in plasmatic individual carotenoids. Mountain cyclist stage produced a significant increase in plasma lutein-zeaxanthin whereas carotene, cryptoxanthin and lycopene did not change. Vitamin E was maintained in plasma after the stage but increased in lymphocytes. A significant increase in plasma MDA and carbonyl concentration both in plasma and lymphocytes have been found. In summary, the vitamin E recommended daily intake did not avoid the oxidative stress increase induced by exercise in plasma and lymphocytes.

Preconditioned fatty livers are tolerant against normothermic post-ischemic injury in rats

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Hepatic fatty change is a well-documented risk factor in reperfusion injury (1). Ischemic preconditioning (PC) appears as an effective strategy to attenuate the damage due to ischemia and reperfusion (2, 3). The aim of the present study was to evaluate wether PC could be effective in an *in vivo* rat model of liver steatosis induced by a choline deficient diet. After a warm cycle of 60 min of lobar selective ischemia followed by 30 min of reperfusion, a consistent appearance of necrosis was reproduced in both normal and fatty livers. Steatosis increased the post-ischemic H_2O_2 concentration, as well as the formation of HNE-protein adducts. PC was found to control both in normal and fatty livers one of the mechanisms involved in the reperfusion injury, i.e. oxidative stress and lipid peroxidation. The marked quenching of oxidant generation and oxidative reactions seem to be one of the pathways by which PC reduces the reperfusion injury and cell death.

(1) Caraceni P; Nardo B; Domenicali M; Turi P; Vici M; Simoncini M, et al. *Hepatology 1999; 29: 1139-46.*

(2) Peralta C; Bulbena O; Xaus C; Prats N; Cutrìn JC; Poli G, et al. *Transplantation 2002; 73: 1203-11*.

(3) Cavalieri B; Perrelli MG; Aragno M; Mastrocola R; Corvetti G; Durazzo M; Poli G and Cutrìn JC. *Liver Transplantation 2002; 8:990-9.*

Ischemic preconditioning attenuates the oxidant-dependent mechanisms of cell damage and death in the reperfused rat liver

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In an *in vivo* rat model of liver ischemia followed by reperfusion a consistent appearance of necrosis and activation of biochemical pathways of apoptosis was reproduced and monitored after 30 minutes reperfusion. The prior application of a short cycle of ischemia-reperfusion (10 min + 10 min) (1) positively conditioned recovery of the organ at reperfusion, attenuating both necrotic and apoptotic events. Preconditioning at least halved cell oxidative damage occurring early at reperfusion. As a major consequence, the increase of cytolysis occurring at reperfusion was about halved by preconditioning. The attenuation of apoptosis afforded by preconditioning also appeared at least partly related to its inhibitory effect on H_2O_2 and HNE production. Both molecular intermediates have been demonstrated to be pro-apoptotic in a number of experimental models. The overall data point to a marked quenching of oxidant generation and oxidative reactions as one major mechanism through which ischemic preconditioning exerts protection against necrotic and apoptotic insult to the post-ischemic liver.

(1) Peralta C; Bulbena O; Xaus C; Prats N; Cutrìn JC; Poli G, et al. *Transplantation 2002; 73: 1203-11.*

Up- and downregulation of β-carotene 15,15'-monooxigenase by β-carotene and retinoic acid in human retinal pigment epithelial cells

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β-Carotene is cleaved into retinal by β-carotene 15,15'-monooxigenase (BCO), which is the key enzyme in the vitamin A synthetic pathway in animals. In human, BCO is expressed in a wide range of tissues including retinal pigment epithelium (RPE). We studied the effect of β-carotene and retinoic acid on BCO mRNA expression and retinol formation in human RPE (ARPE-19). Cells were incubated with medium containing different concentrations of *all-trans*- β -carotene (0,5µM to 40µM) or *all-trans*-retinoic acid (0.5µM to 10µM). BCO mRNA was quantitated using SYBR green real time quantitative PCR. Levels of β-carotene and retinol were measured by HPLC. We found a two-fold increase of BCO mRNA expression in cells incubated with 5µM β-carotene after 24 hours and a three-fold decrease in cells incubated with 1µM retinoic acid after 48 hours. The uptake of β-carotene was increased linearly in parallel to the concentration in the medium, *i.e.* a maximum uptake at a 40µM concentration. Retinol levels were increased two-fold in the cells incubated with 5µM β-carotene after 6 days. These findings suggest that β-carotene can serve as a precursor for vitamin A in RPE cells and that β-carotene metabolism is controlled by retinoic acid at the transcriptional level.

Differential zonal distribution of hydrogen peroxide, ascorbate and antioxidative enzymes in *Allium cepa* roots

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In onion (*Allium cepa* L.) roots reactive oxygen species (ROS) are produced mainly at the cell wall and at outer side of the plasma membrane. One of these ROS, hydrogen peroxide, was detected by cytochemistry and was found at different proportions depending on the root zone. In meristematic and elongation zones a high number of plasma membranes, cell wall middle lamella and intercellular spaces showed H_2O_2, which decreased drastically towards the root base. The distribution of intracellular (symplastic) antioxidant ascorbic acid was similar to that found for H_2O_2, being the reduced form ascorbate (ASC) significantly higher than the oxidized one, dehydroascorbate (DHA). Nevertheless, external (apoplastic) ascorbic acid only represented 2-8% of the whole content, being DHA the predominat form. The antioxidative enzymes ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase, catalase and glutathione reductase showed different activity patterns depending on the zone of the root and their apoplastic or symplastic origin. The differential zonal balance of these molecules and the cooperative role of hydrogen peroxide and other ROS with the ascorbate system on cell division, elongation and differentiation in onion roots are discussed.

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In vitro antioxidant profile of polyphenols

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More than fifty polyphenolic compounds were tested in three antioxidant assays, i.e., the $DPPH^{\cdot}$ and $HO^{\cdot}$ scavenging assays and the microsomal lipid peroxidation assay, in order to evaluate their *in vitro* antioxidant profile. Since a promising antioxidant compound should show a lipid peroxidation-inhibiting activity at micromolar level and a low cytotoxicity, the cytotoxicity of the phenolic compounds was also studied. Finally, some active compounds were evaluated for their complement inhibiting properties.

Interesting polyphenolic compounds showing a wide range of antioxidant activities were procyanidin C1, caffeic acid anilides, caffeic acid dopamine amide, gallic acid, and quercetin. However, gallic acid exhibited a high cytotoxicity, while for quercetin a prooxidant activity was demonstrated in an EPR system. (+)-Catechin and kaempferol were relatively strong inhibitors of lipid peroxidation and showed no cytotoxicity at relatively high concentrations, but they exhibited a low activity in both the free radical scavenging and complement inhibiting assays.

Finally, genistein exhibited a very low antioxidant activity in both the lipid peroxidation and the $DPPH^{\cdot}$ scavenging assay, a high cytotoxicity, and a low complement-inhibiting activity, and can therefore not be classified as an interesting antioxidant *in vitro*.

Further investigation is needed to evaluate their *in vivo* antioxidant profile.

In vitro antioxidant profile of polyphenols

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More than fifty polyphenolic compounds were tested in three antioxidant assays, i.e., the $DPPH^{\cdot}$ and $HO^{\cdot}$ scavenging assays and the microsomal lipid peroxidation assay, in order to evaluate their *in vitro* antioxidant profile. Since a promising antioxidant compound should show a lipid peroxidation-inhibiting activity at micromolar level and a low cytotoxicity, the cytotoxicity of the phenolic compounds was also studied . Finally, some active compounds were evaluated for their complement inhibiting properties.
Interesting polyphenolic compounds showing a wide range of antioxidant activities were procyanidin C1, caffeic acid anilides, caffeic acid dopamine amide, gallic acid, and quercetin. However, gallic acid exhibited a high cytotoxicity, while for quercetin a prooxidant activity was demonstrated in an EPR system. (+)-Catechin and kaempferol were relatively strong inhibitors of lipid peroxidation and showed no cytotoxicity at relatively high concentrations, but they exhibited a low activity in both the free radical scavenging and complement inhibiting assays.

Finally, genistein exhibited a very low antioxidant activity in both the lipid peroxidation and the $DPPH^{\cdot}$ scavenging assay, a high cytotoxicity, and a low complement-inhibiting activity, and can therefore not be classified as an interesting antioxidant *in vitro*.
Further investigation is needed to evaluate their *in vivo* antioxidant profile.

Individual Variability in Stress-Related Gene Expression in Adult and Older Subjects in Response to Sodium Selenite Supplementation

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Dietary selenium intake in the UK has fallen over the last 20 years. The functional consequences of this reduced intake are unknown but may contribute to impaired immune function, an increased risk of some cancers, and an increased susceptibility to viral disease. The situation in older subjects may be more marked. Individual requirements for dietary selenium have not been defined but some data indicates that there may be a wide variability in responses of UK subjects to small supplemental doses of selenium. This study has examined the individual variability in stress-related changes in gene expression in adult (20-50 y) and older (>65 y) subjects in response to selenium supplementation (100µg/day as sodium selenite). An adult placebo group was also included in the study. Using Atlas™ Stress cDNA micro-arrays we observed significant changes in stress-related gene expression following selenite supplementation in adult subjects. In general, genes encoding stress-activated protein kinases (SAPK), antioxidant enzymes, DNA repair proteins and enzymes, and heat shock protein 70 (HSP70) were decreased. These changes in gene expression showed some individual variability with occasional adult subjects showing no changes or an increased expression. The individual variability was greater in older subjects such that consistent changes in gene expression in response to selenite supplementation were not observed. No significant changes in gene expression were observed in the adult placebo group. These data indicate that there is a wide variability in responses of adult subjects to selenium supplementation with this variability being more marked in older subjects. The increased expression of stress-related genes in a small number of subjects also suggests that selenite may have some minor negative effects and these subjects may benefit from a lower supplemental dose of selenium.

Oncolyn Neutralizes the Oxidative Damage by Asbestos in vitro, in vivo, and in Patients

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Asbestos can cause pulmonary injury, as well as fibrosis and mesothelioma; and that alveolar macrophages play an important role in these processes. The disorder of oxidation and antioxidation may contribute to the process of fibrosis and mesothelioma caused by asbestos. Oncolyn® is a formulated extract from three edible plants containing polyphenols, proanthocyanidins, plant saponins and other natural plant ingredients in synergistic combination with effective antioxidants and cytoprotective function. The current study investigated the effect of Oncolyn on the neutralization of oxidative damage in alveolar macrophages, both in vitro and in rats and used in patients with confirmed Mesothelioma. The DNA strand breakage, nitric oxide (NO) generation and, superoxide dismutase (SOD) activity, glutathione peroxidase (GSH-Px) activity, and NO synthesize enzyme were measured in alveolar macrophages treated with asbestos and Oncolyn.

In blood and lung tissue of Wistar rats exposed to asbestos, the interaction between inhaled asbestos and membranes of alveolar macrophage (AM) is an important event in the development of fibrosis. Asbestos can injure alveolar macrophages directly, and can also induce them to release a variety of mediators which play a key role in the initial lung injury. Alveolar macrophages obtained from Wistar rats cultured with asbestos and Oncolyn for one or two hours. Results from analysis by Comet assay demonstrated that the percent of Comet cells decreased, and the length of DNA migration became shorter, in alveolar macrophages treated with Oncolyn as compared to alveolar macrophages receiving no Oncolyn. Oncolyn also inhibited nitric oxide generation, as measured by the amount of nitrite released into the culture medium. NO synthesize enzyme in soluble extracts of alveolar macrophages treated with Oncolyn for 2

hours and 4 hours was significantly lower than that in macrophages without Oncolyn treatment. Further, as the concentration of Oncolyn increased, superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities correspondingly increased.

An in vivo model of asbestos pulmonary damage was produced by a single administration into bronchi of an asbestos solution (10 mg/ml) to Wistar rats. Following the administration, Oncolyn was given to rats for 40 days, and then the rats were sacrificed. Blood and lung tissue were collected to examine the content of NO and the activities of NOS, SOD, GSH-Px, MDA, CAT, GST and by histopathology. The changes in histopathology of lung tissue were measured. The content of NO and the activity of NOS significantly fell in Oncolyn-treated group when compared with the group receiving asbestos alone. In lung tissue, the activities of antioxidative enzyme is high when asbestosis rats were treated with Oncolyn. The lungs of Wistar rats without Oncolyn treatment showed early confluent bronchopneumonia in contrast with rats treated with Oncolyn which only exhibited mild resolving acute and chronic alveolitis. Many patients with confirmed diagnosis of mesothelioma extended their life expectancy and one patient is in clinical remission for four years and working full time as of January, 2003.

Can insulin have a neuroprotective role in cultured cortical neurons submitted to oxidative stress?

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Insulin has been suggested to have a neuroprotective role in neurodegenerative disorders, such as Alzheimer's disease, in which oxidative stress was previously demonstrated. In this study, we investigated the role of insulin under oxidative stress conditions, induced by the oxidizing pair ascorbate/Fe^{2+}, in cultured cortical neurons. A significant decrease in cell viability, determined by the MTT assay, was observed after the induction of oxidative stress (15 min), followed by 5 h-postincubation in Neurobasal medium. Under these conditions, we have studied the effect of insulin (0.1, 1, 10 and 25 μM), which was added to the culture medium 96 or 48 h prior and/or during the induction of oxidative stress. Preincubation of cortical neurons for 96 h with increasing concentrations of insulin induced an U-shaped curve in MTT reduction, and 25 μM insulin prevented the effect of oxidative stress. Insulin (0.1 and 10 μM, added 48 h prior and during the incubation with ascorbate/Fe^{2+}, respectively) protected against oxidative stress-mediated decreased in MTT reduction. Furthermore, insulin (0.1, 10 and 25 μM, incubated for 96 or 48 h) significantly decreased the levels of lipid peroxidation induced by ascorbate/Fe^{2+}. These results suggest that the neuroprotective role of insulin in the brain may be associated with a protective effect against oxidative stress-mediated cell damage.

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Sulindac scavenging activity against reactive oxygen and nitrogen species. A comparative study with its metabolites sulindac sulfide and sulindac sulfone

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Sulindac is a sulfoxide prodrug that *in vivo* is converted to the metabolites sulindac sulfide and sulindac sulfone. It is therapeutically used for anti-inflammatory and analgesic effects in the symptomatic treatment of acute and chronic rheumatoid arthritis, osteoarthritis, and ankylosing spondylitis. In addition to its anti-inflammatory properties, sulindac and its metabolites have been shown to have an important role in the prevention of colonic carcinogenesis. Although the inhibition of prostaglandin synthesis constitutes the primary mechanism of action of sulindac, it is well known that reactive oxygen species (ROS) and reactive nitrogen species (NOS) are implicated in the pathophysiology of inflammation and colon cancer. Thus, the aim of this study was to evaluate the scavenging activity of sulindac and its sulfone and sulfide metabolites against an array of ROS ($HO^{\cdot}$, $O_2^{\cdot -}$, HOCl) and RNS ($^{\cdot}NO$ and $ONOO^-$) using various in vitro systems. The obtained results demonstrate that the metabolism of sulindac increases its scavenging activity for all NOS and ROS studied, notably in what concerns to the scavenging of HOCl. These effects may strongly contribute for the anti-inflammatory and anticarcinogenic efficacy that has been shown for sulindac.

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Antioxidant defense of *Trypanosoma brucei*

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Antioxidant defense in T. *brucei brucei*, the causative agent of the African Nagana disease, depends on a peroxiredoxin-type peroxidase (*Tb*TXNPx) that is reduced by a thioredoxin-related protein, tryparedoxin (*Tb*TXN). TXN is reduced by trypanothione [$T(SH)_2$], which is provided by *de novo* synthesis from spermidine and GSH or regenerated by trypanothione reductase. A gene obtained from *T. brucei* DNA by PCR encoded trypanothione synthetase that catalysed both steps of $T(SH)_2$ synthesis. *Tb*TXN and *Tb*TXNPx were obtained analogously. *Tb*TXNPx proved to be a cytosolic-type TXNPx that is weakly active with and easily inactivated by lipid hydroperoxides. Its kinetic pattern suggested negative cooperativity between subunits. MALDI-TOF analysis, gel permeation, and electron microscopy revealed that oxidized *Tb*TXNPx forms decameric rings and higher aggregates thereof. Reduction resulted in pearl chain-like structures of lower MW. A C43S mutation of *Tb*TXN yielded a dead-end intermediate with *Tb*TXNPx that mimics a catalytic one. Its analysis revealed that *Tb*TXNPx in its intermediary oxidation state is again decameric and that the decameric rings possess ten substrate-accessible reaction centers. The data thus reveals major conformational changes associated with the catalytic cycle of *Tb*TXNPx and further underscores species-specific peculiarities of the system.

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Initial studies on the effect of the antitumor agent dequalinium in leukemic cells

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Delocalized lipophilic cations, such as dequalinium (DQA), comprise a new class of antitumor agents which accumulate in mitochondria targeted by the negative electric potential across the mitochondrial membrane, being selectively more toxic for tumor than for normal cells. Mitochondria make an integral contribution on the regulation of main aspects of cell biology such as energy production, molecular metabolism, redox status, calcium signalling and programmed cell death. These compounds have been related with diverse alterations of the cell homeostasis. It has been suggested that DQA induces cell death of neurones by a mixture of apoptosis-necrosis related to the oxidative stress and ATP depletion, being the basis for cytotoxicity. To understand the antitumor properties of DQA, we are studying its effect on two human leukemia cell lines: K562, derived from a chronic myeloid leukemia in blastic crisis, which is very resistant to treatments that induce apoptosis in other myeloid leukemia cells, and the line NB4, derived from acute promyelocytic leukemia. We are studying cell cytotoxicity, cell cycle and apoptosis-necrosis caused by DQA. Preliminary results indicate that low doses of DQA induce both apoptosis and arrest of proliferation in NB4 cells, without apparent apoptosis in K562 cells. Higher doses mainly induce cell death by necrosis. These studies will open future possibilities of application in cancer chemotherapy as well as an extension of its application towards new perspectives related to DQA as a vector for mitochondrial gene therapy.

Evidence for a novel neuroprotective role for peroxynitrite through activation of the pentose phosphate pathway

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Peroxynitrite is thought to be a nitric oxide-derived neurotoxic effector molecule involved in the disruption of key energy-related metabolic targets. In order to assess the consequences of such interference on cellular glucose metabolism and viability, here we studied the possible modulating role played by peroxynitrite in glucose utilization in neurons in primary culture. We report that peroxynitrite triggers a rapid stimulation of pentose-phosphate pathway (PPP) activity and prevents nitric oxide-mediated NADPH depletion, glutathione oxidation, and apoptotic cell death in neurons. The biochemical analyses performed strongly suggest that peroxynitrite activates glucose-6-phosphate dehydrogenase (G6PD), an enzyme that catalyses the first rate-limiting step of the PPP. Moreover, functional overexpression of the G6PD gene in stably-transformed PC12 cells induced NADPH accumulation and showed remarkable resistance to nitric oxide-mediated apoptosis, whereas G6PD-targeted antisense inhibition depleted NADPH levels and exacerbated cell vulnerability. In the light of these results, we suggest that peroxynitrite could have a novel neuroprotective signaling function aimed at preventing the propagation of neurodegeneration.

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Oxidative stress in biological systems: prediction of redox mechanisms through electrochemistry

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Free radical generation is directly related with oxidation processes in biological systems being reactive oxygen and nitrogen species implicated in a variety of biological phenomena such as mutation, carcinogenesis, degenerative diseases and aging.

Direct detection of free radicals is a difficult task mainly because these chemical entities are short-lived and highly reactive. Thus, oxidative damage is often evaluated by measurement of secondary products of oxidation.

Electrochemical studies could furnish an enormous amount of evidence regarding the mechanisms of biological electron-transfer processes. Moreover, they could be used as an important basis to get information for the establishment of oxidative profiles of drugs and metabolites including the prediction of their oxidative pathways.

In this work some examples of the usefulness of electrochemical methods in the study of oxidative stress phenomena will be presented.

Cell type dependence in NF-κB activation by oxidative stress

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NF-κB is a redox-regulated transcription factor involved in many cellular processes, including regulation of immune and inflammatory genes, apoptosis, cell proliferation and development. Several reports have recently shown that the mechanism of NF-κB activation by an oxidative stress (including the tyrosine phosphatase inhibitor pervanadate and hypoxia/reoxygenation) was totally distinct from those triggered by proinflammatory cytokines or mitogens; it involves tyrosine phosphorylation of the inhibitor IκBα without activation of the IκB kinase (IKK) complex and phosphorylation of serine 32 and 36. In the present work, we provide several lines of evidence that NF-κB activation by an oxidative stress is not tyrosine phosphorylation-dependent in every cell lines. By using the T lymphocytic cell line CEM, we showed that pervanadate treatment activate NF-κB through tyrosine phosphorylation of IκBα without degradation of the protein. This phosphorylation does not implicate the IKK complex. In contrast, treatment with hydrogen peroxide does not involve tyrosine phosphorylation of IκBα, but induces a strong activation of the IKK complex, leading to phosphorylation of serine 32 and 36, degradation of IκBα and nuclear translocation of NF-κB. In another T lymphocytic cell line (Jurkat), hydrogen peroxide activate NF-κB via the same mechanism that pervanadate, *i.e.* tyrosine phosphorylation of IκBα without IκB kinase activation. Our findings suggest that the mechanism of NF-κB activation by oxidative stress is cell type-dependent, implicating either a tyrosine phosphorylation-dependent mechanism, or a classical mechanism involving IKK complex activation.

Xanthine oxidase inhibition with allopurinol prevents ERK1/2 and p38 phosporylation and NF-kappaB activation in rats exercised until exhaustion

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Xanthine oxidase is an enzyme involved in oxidant production and muscle damage during exhaustive physical exercise (1). Recent studies show that there is a redox regulation of cellular signalling and that the generation of reactive oxygen species leads to the activation of MAP-kinase pathway (2). It has been shown that this pathway induces the activation of the redox-sensitive transcription factor NF-κB which plays an important role in the regulation of gene activity (3).

Thus, the purpose of the present study was to test the hypothesis that free radical production in exhaustive physical exercise causes an activation of important cell signalls as MAPKs and NF-kappaB and the effect of allopurinol administration (an inhibitor of the xanthine oxidase).

Male wistar rats (age 8 weeks) were randomly divided into 3 groups: control group (C), exercised until exhaustion group (E) and exercised until exhaustion group but pretreated with 32 mg/Kg of allopurinol (EA). Xanthine oxidase activity was measured in plasma. Oxidized and reduced glutathione were measured in blood and in gastrocnemius muscle homogenate as an index of oxidative stress. Western Blot analysis was used to measure MAPKs phosphorylation. Gel mobility shift assay was used to measure NF-κB activation.

Our results show that the inhibition of xanthine oxidase with allopurinol prevents glutathione oxidation, MAPks phosporilation and NF-kappaB activation in gastrocnemius muscle of rats after running until exhaustion.

Signals that activate hepatic exercise responses: Oxidative stress and induction of HSP70s

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The liver plays an essential role during exercise, particularly as the main source of glucose for the contracting muscle. The cellular stress response induced by exercise is considered to be the main defence mechanism to preserve cell integrity and functionality in the liver, particularly during intense or prolonged exercise. Oxidative stress could be the most relevant signal to trigger the cellular stress response to exercise in the liver. We tested this hypothesis by studying the kinetics of synthesis and accumulation of HSP70s following a single exercise bout and analysing their correlation with changes in lipid peroxidation indices (free and protein-bound TBARS). With the exception of the constitutively expressed protein HSP73, all HSP70s accumulated early after a single exercise bout. Synthesis rates of HSP70s increased transitorily following induction of their mRNAs. HSP72, the most inducible of all HSP70s, showed two further peaks of accumulation later in the post-exercise period (at 8 and 48 h) in the absence of detectable mRNA. Although the hepatic levels of both free and protein-bound TBARS, showed a trend towards increasing after exercise, their changes did not reach statistical significance. Despite this fact, the accumulation of HSP72 in the liver immediately after exercise correlated directly with the levels of bound TBARS (0.642, $p<0.001$), indicating that the induction of HSP72 sensitively follows changes in oxidative stress. Altogether, the reported results suggest that the early, transcriptionally-controlled induction of synthesis and the accumulation of HSP70s represent preliminary steps of a series of HSP70-related events with which liver cells react against exercise-induced oxidative damage.

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Molecular variants of the hepatic cytosolic HSP70s: Relevance for the exercise response

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The cytosolic members of HSP70s, the constitutively expressed (HSP73) and the highly stress inducible (HSP72) proteins, display at least 3 isoelectric point variants. In non-stressed animals the hepatic HSP72 levels were very low and only one of the variants was detected. In exercised animals, when all variants were present, the two-dimensional patterns were similar for both proteins: a major variant (1) flanked by one more acidic (2) and a second more basic (3). The functional relevance of these variants for the hepatic exercise response was investigated by studying their presence and relative abundance following a single exercise bout, using the major variant of the constitutively expressed protein as an internal reference. The acidic variants of both proteins, $HSP72_2$ and $HSP73_2$ increased transitorily shortly after exercise. In contrast with $HSP73_1$, that did not change, the abundance of $HSP72_1$ increased during the early post-exercise and its acidic variant, $HSP72_2$, increased faster initially (as shown by the $HSP72_2$-to-$HSP72_1$ ratio). This ratio was also high in the peaks of HSP72 accumulation at 8 and 48 h post-exercise. Incubation of whole liver protein homogenates with, or without added, alkaline phosphatase or calcineurin increased the proportion of the basic variants of both proteins by 10-15 % and reduced that of the acidic ones. Since these transitions were inhibited by adding a phosphatase inhibitor mix, the data indicate that the generation of variants of the HSP70s involves phosphorylation/dephosphorylation processes. However, due to the relatively small fraction of basic variant that was recovered following phosphatase treatment, it is difficult to assume that this is the only mechanism responsible for generating HSP70 variants.

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Induction of vascular endothelial growth factor (vegf) and vascular endothelial growth factor receptor 2 (vegfr2) by oxidative challenge: Mechanisms and implications in endothelial protection

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Exogenous H_2O_2 induces a different response on endothelial cells in culture (EC) depending on concentration, namely, low concentrations (0.5 µM) were mildly cytoprotective, whereas concentrations > 100 µM were cytotoxic. H_2O_2 induces VEGF expression, which has a critical role in cytoprotective actions. The objectives of the present study were: 1) to examine the mechanisms of effect of H_2O_2 on VEGF receptors expression (VEGFR1 and VEGFR2); 2) To investigate the signalling pathways and transcription factors implicated in the induction of VEGF expression. With this aim, we stimulated EC with different concentrations of H_2O_2 (0.05–500 µM, 30 min). After 24 h, VEGFR2 mRNA expression was increased only at 250–500 µM H_2O_2, while VEGF expression was increased at 0.05–2 µM concentrations. The VEGFR1 expression was not affected in any condition. In the presence of exogenous VEGF, VEGFR2 mRNA expression was increased. However, the H_2O_2–induced VEGFR2 expression did not change in the presence of an anti-VEGF antibody. To examine the pathways involved in the stimulation of VEGF mRNA expression by H_2O_2, the EC were treated with 0.5 and 250 µM H_2O_2 in the presence of several inhibitors of VEGF or ROS-related signalling pathways. The VEGF expression decreased strongly in the presence of genistein, LY294002, calphostin C, and N-AcetylCys. Furthermore, a stimulation by H_2O_2 (0.5 and 250 µM) of a set of transcriptional factors, namely, Sp-1, NFκB and HIF-1, was detected by EMSA. Conclusions: In EC, oxidative stress by H_2O_2 induces VEGFR2 expression, in a not ligand-regulated manner. The autocrine VEGF expression is regulated by diverse mechanisms and depends on at least 3 signalling mechanisms, i.e., tyrosine phosphorylation,

PI3K/Akt and PKC, and is regulated by the availability of SH. The exposure of EC to H_2O_2 stimulates the activation of 3 transcription factors, i.e., Sp-1, NFκB and HIF-1.

Ascorbic acid decreases uva-induced nitric oxide formation in human dermal fibroblasts

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The role and importance of reactive nitrogen intermediates such as nitric oxide (NO) and peroxynitrite in response to skin damage and photoaging is still unclear. Both protective and cytotoxic properties of NO have been described. Thereby ascorbic acid with its antioxidative characteristics might be engaged in NO formation in the skin.

Cultivated fibroblasts were grown to confluency on glass slides and supplemented with a single dose of ascorbic acid (25, 50 and 100 µM) for 4 days. Cells were then irradiated with a suberythemal dose of 20 J/cm^2 UVA. NO levels were measured at 1.5, 2, 4, 6, 8, 10, 12, 24 and 48 h post irradiation (p.i.) and compared to non-irradiated cells. We measured NO formation directly in living cells by a fluorescent indicator, DAF-2 DA using a digital fluorescence imaging system.

NO formation in supplemented fibroblasts generally decreased with increasing ascorbic acid concentrations in contrast to control cells. Control cells show a peak of NO formation 2 h p.i. and a further increase with peak value at 24 h p.i.. The increase of NO formation 2 h p.i. was dose dependently lowered with increasing ascorbic acid concentration. Any further effects were totally suppressed. In conclusion ascorbic acid seems to suppress NO formation in human fibroblasts after UVA treatment.

Testis antioxidants in fish steroidogenesis

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It is known testis have a high content of polyunsatured fatty acids on their cell membrane. This characteristic renders the gonads prone to the deleterious effects of reactive oxygen species. Although the excessive production may have deleterious effects, the controlled release or scavenging of some reactive oxygen species appears to modulate their reproductive functions. To our knowledge, the different testis antioxidant systems in fish have not been extensively studied. Our goal was to measure testis glutathione peroxidase and vitamin E levels during the different phases of gonadal development in the sea bass, *Dicentrarchus labrax*, collected in the Gulf of Naples. Glutathione peroxidase was assayed enzimatically, vitamin E using HPLC, and the steroidogenetic status was defined histologically and by sex steroid hormone RIA. Both antioxidants were present in the sea bass testis with different steroidogenetic patterns. Testis vitamin E concentration resulted parallel to the androgen levels, and increased during the gonadal recrudescence. In contrast, glutathione peroxidase was expressed at low levels, which did not exhibit any specificity. We detected changes in testis vitamin E very similar to those we found in plasma vitamin E levels during previous studies. These data can be explained, like in mammals, with a specific vitamin E role in the steroidogenesis facilitation, tissue remodeling and synthesis of collagen, all events attending the testis-cycle.

Lowering of NO stores in patients with cardiovascular risk factors and endothelial dysfunction

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Background: Endothelium-derived nitric oxide (NO) not only acts regionally but may also exert systemic effects by formation of nitosylated species (RNO) with a long half-life. S-Nitrosothiols in plasma represent a significant proportion of total RNO. Here we tested the hypothesis whether or not endothelial dysfunction in patients with cardiovascular risk factors is associated with a lowered concentration of S-nitrosothiols (RSNO) which could be used as a blood test for endothelial dysfunction.

Methods: The investigation was carried out on 43 patients with 1-4 major cardiovascular risk factors (RF) and on 8 healthy age-matched control-subjects (C). Venous blood samples were analyzed for nitrite concentration in plasma using the Griess method in a flow-injection assay (FIA). RSNO was determined after preincubation with $HgCl_2$ (8,9mmol/L). Endothelium-dependent dilation was quantified noninvasively by FMD using standard techniques.

Results: In C RSNO concentration was 682 ± 109nmol/L. In patients with RF NO-stores were significantly lowered with increasing number of total RF: 452 ± 76 > 404 ± 41 > 368 ± 79 > 141 ± 40nmol/L ($p < 0,01$ vs C). FMD was diminished in parallel: 11,4 ± 0,9 > 5,5 ± 1,3 > 3,0 ± 0,6 > 2,9 ± 0,6 > 2,2 ± 0,6% ($p < 0,01$). There was a positive correlation between FMD and RSNO ($R= 0,51$, $p < 0,01$). RSNO concentrations determined by FIA are more than 10-fold higher than levels encountered with a chemiluminescence assay.

Conclusion: Endothelial dysfunction is associated with a lowering of circulating bioactive NO in human blood. Measurement of RSNO concentration could be used as biochemical marker for endothelial dysfunction. However, absolute levels of RSNO have to be clarified.

PON1 paraoxonase activity is reduced during HDL oxidation and with aging and is an indicator of HDL antioxidant capacity

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High-density lipoproteins are antiatherogenic molecules, whose protective action is due in part to the associated enzyme, paraoxonase (PON1). PON1 antioxidant activity might be altered, in vivo, during oxidative stress, which could affect the antiatherogenic properties of HDL. The aim of this study was to investigate the effect of HDL oxidation on PON1 paraoxonase activity and of the antioxidant protection of LDL oxidation by HDL. Also we were interested in investigating of the PON1 activity with aging.

HDL oxidation was induced by incubation with THP1 cells, with copper ions or induced by $^{\cdot}OH$ and $O_2^{\cdot -}$ oxygen free radicals produced by gamma-radiolysis. HDL oxidation was followed by the measurement of lipid peroxide formation, and PON1 activity was determined by measuring the rate of paraoxon hydrolysis.

Our results show that HDL oxidation is accompanied by a reduction in the PON1 paraoxonase activity. The extent of PON1 inactivation depends both on the extent of HDL oxidation and on the oxidation system used. The rates of HDL oxidation and PON1 inactivation were significantly correlated ($r = 0.93$ $p < 0.0054$). The antioxidant action of HDL towards LDL oxidation and the degradation of PON1 paraoxonase activity were significantly correlated ($r = 0.95$, $p < 0.04$). Plasma as well as HDL PON1 activity decreased significantly with aging.

Reduction of PON1 activity could be a non-negligible factor of increased incidence risk to atherosclerosis and related diseases with aging.

The possible role of $NADP^+$-dependent isocitrate dehydrogenase in antioxidative defense and in aging

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NADPH is a reducing equivalent for the NADPH-dependent thioredoxin system and the regeneration of reduced glutathione, critically important in cellular defense against oxidative damage. Little information is available about the role of cytosolic and mitochondrial $NADP^+$-dependent isocitrate dehydrogenases (ICDH) in antioxidant defense. In this study we investigated the role of ICDH against cytotoxicity induced by oxidative stress by comparing the relative degree of cellular responses in three different NIH3T3 cells with stable transfection with the cDNA for mouse cytosolic or mitochondrial ICDH in sense and antisense orientations. Upon transient exposure to increasing concentrations of H_2O_2, menadione or radiations, the cells with low levels of ICDH became more sensitive to oxidative insult. Lipid peroxidation, oxidative DNA damage, and intracellular peroxide generation were higher in the cell-line expressing the lower level of ICDH. This study provides direct evidence correlating the activities of ICDH and the maintenance of the cellular redox state, suggesting that ICDH plays an important role in cellular defense against oxidative stress. The possible role of ICDH, presumably via the antioxidative effect, in aging was also examined in IMR-90 human diploid fibroblast cells and in rats.

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Influence of lectins *in vitro* on the free radical processes of erythrocyte membranes in case of diabetes

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Lectins are proteins capable of connecting various carbohydrate determinants. It is know that in case of pathological process development there are changes of cell membranes carbohydrate determinants.

The aim of this investigation: was the study structural in erythrocyte membranes in children with type 1 diabetes according to the duration of illness and influence of lectins on the free radical processes. The patients were divided in 3 groups according to the duration of the illness: the 1st group consisted of children in which the period of manifested diabetes lasted no longer that 1 year; the 2nd group consisted of children with duration of the illness from 2 to 5 years; and the 3rd group consisted of children with the duration of illness from 7 to 12 years. Control group consisted of 45 age-matched normal subjects.

We have used the following preagglutination concentration of lectins: the β-galactose specific, peanut lectin, PNA (125 mkg/ml), the N-acetyl-D-glucosamine specific, wheat lectin, WGA (6 mkg/ml), the mannose specific, Canavalia lectin, ConA (125 mkg/ml) and the Bacillus polymyxa cell surface lectin, L II (1,4 mkg/ml) specific for D-galactosamine, glucuronic acid, fructose-1,6- diphosphate, D-glucosamine.

It was shown that after incubating lectins PNA, LII, ConA with erythrocytes in the control group of children the intensity of chemiluminescence (Imax) has immediately increased in comparison with native erythrocytes (1 mV) and has made up 2,2; 2,0; 1,4 mV correspondingly. It is interesting to point out that, in process of incubating erythrocytes with WGA activation of free- radical processes did not take place and that was proved by the absence of reliable difference in Imax in comparison with native erythrocytes. In the groups of sick children WGA makes I max 2,3 times higher in the 1st group and 5,5 times higher in the 2nd group. We observed

the similar kind of influence concerning PNA and LII as well. In the 1st group PNA makes Imax 2,5 times higher and LII makes it 3,3 times higher in comparison with native erythrocytes. In the 3rd group of patients PNA and LII make Imax 3,1 and 4,3 times higher. In the 2nd group of patients we have discovered the change of I max in comparison with the 1st group and the 3rd group in the process of incubating erythrocytes with PNA, WGA, LII. The given lectins caused the decrease of Imax and this parameters value reached the control quantity. When we incubated ConA with erythrocytes of children with IDDM, the increase of Imax occurred in all the 3 groups: a 1,2-fold increase in the 1st group; a 1,8-fold increase in the 2nd group and a 2-fold increase in the 3rd group compared to the control group (native erythrocytes). The desialisation process is the factor of the structural change in the cell receptors under the insulin deficit conditions. As a result, the receptor specificity for the exogenous ligand (lectins) is changed. Thus, the research that has been carried out makes it possible to talk about the capability of carbohydrate connecting proteins- lectins to manifest the changes of carbohydrate determinants on the erythrocytes membrane at various stages of diabetes and to cause changes in metabolic processes as a result of connecting with various receptors.

Cocoa Extract is a Rich Ingredient in Flavanols and Flavonols Compounds

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Cocoa is not only a pleasant taste food it can be also considered a healthy food thanks to its rich content in phenolic compounds The cocoa extract obtained from cocoa may constitute a functional ingredient very interesting for food industries. The aim of our study was to identify and quantify the phenolic composition in a cocoa extract. Liquid chromatography coupled with ionspray mass spectrometry in the tandem mode (LC/MS/MS) was used. The extract resulted very rich in phenolic compounds, eleven monomeric compounds have been identified and quantified in the extract and also two dimers. The major polyphenolic compounds were the flavanols epicatechin (2.39 mg/g) and procyanidin B2 (2.26 mg/g), followed by catechin (0.91 mg/g) and procyanidin B1 (0,27 mg/g). Moreover, this extract is also rich in flavonols which have high antioxidant activity. The major one is quercetin. These results demonstrate that the extract of cocoa is an ingredient plenty of possibilities as a functional one due to its rich content in phenolic compounds. Thus, food companies could enrich with phenolic compounds their food products.

Molecular study of the peroxisomal monodehydroascorbate reductase (MDHAR) of pea plants

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In plant cells, ascorbate is a major antioxidant which is involved in the ascorbate-glutathione cycle [1]. The regeneration of reduced ascorbate in this cycle is achieved by the enzyme monodehydroascorbate reductase (MDHAR; EC 1.6.5.4), using NAD(P)H as electron donor. MDHAR activity has been detected in chloroplasts, mitochondria, cytosol and also in peroxisomes [1,2]. However, until now no cDNA has been clearly identified for a peroxisomal MDHAR.

To study the peroxisomal MDHAR in leaves of pea plants (*Pisum sativum* L., cv. Phoenix) several approaches were used. By immunogold electron microscopy the localization of MDHAR in leaf peroxisomes was corroborated. Using a pea cDNA which contains a putative peroxisomal targeting signal PTS1 [3], a histidine-tagged MDHAR was expressed in *E. coli* and purified to carry out the biochemical characterization of this enzyme. Finally, genomic analysis was applied and the tissue expression of MDHAR in pea plants was studied.

These molecular data represent a first step to achieve the full characterization of this important enzyme of the peroxisomal antioxidative metabolism.

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Reduction of Tobacco Specific Nitrosamines (TSNAs) by Increasing Native Antioxidants in Tobacco

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Tobacco-specific nitrosamines (TSNA) are formed during curing and are derived from the reaction of a reactive nitrosating species with the tobacco alkaloids. The presence of antioxidants in tobacco may inhibit the nitrosating reaction, thus reducing the amount of TSNA formed. Therefore, the amounts of antioxidants present and the location within the cell at time of nitrosation may be more critical to TSNA inhibition. We have investigated the status of TSNA accumulation and the dynamics of the endogenous antioxidants during curing. Total antioxidant capacity of tobaccos was determined for the first time using ferric reducing/antioxidant power assay. Tobaccos cured to different extents contained widely differing antioxidant capacities which were well correlated with their total polyphenol concentration. The amount of endogenous antioxidants present in tobacco leaves is inversely correlated with the TSNA concentration. The finding supports the idea that higher endogenous antioxidants present during the senescence and curing of tobaccos may affect the propensities of those tissues to inhibit TSNA formation by reacting with nitrite or nitrosating species, or both. Experiments that raised burley leaf antioxidant capacity by stressing tobacco plants resulted in leaves with increased antioxidant capacity (about 2-fold) and reductions of TSNA of up to 90 % as compared to untreated control plants. We hypothesize that, under stress conditions, the capacity of the plants to assimilate CO_2 is reduced, and the photosynthetic electron flux to O_2 increases, thus resulting in the increased production of active oxygen species. The burst of active oxygen species may serve as a signaling agent to activate antioxidant defense mechanisms.

Antioxidant activity of polyphenols in carob bean

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The carob tree (*Ceratonia siliqua* L.) is considered one of the characteristic trees in Mediterranean countries. The carob fruit is brown in colour and its principal components are the pod and the seeds. The commercial value of the carob bean is due to the endosperm of the seeds, as carob gum (galactomannans). Pods and the rest of the seeds, cuticle and germ, are considered by-products and have a very poor economic value. Carob pods have important sugar content (40-50%) and carob germs have a high content of protein (40-50%).

Pods and germs polyphenols were extracted, separately, with organic solvents. The extracts were used to measure the antioxidant activity. The free radical scavenging activity was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH·) method. Vitamin E was used as standard and compared with the extracts in terms of the antioxidant activities in DPPH· method. The amount of sample necessary to decrease by 50% the initial DPPH· concentration (EC_{50}) was determined for all the samples. The results demonstrated that 70% acetone extracts showed the highest scavenging activity. The antioxidant activity of 1g of the extracts expressed as the capacity of scavenging free radicals is equivalent to 122 mg (pods), 91 mg (germ) and 909 mg (germ with cuticle) of vitamin E. In all cases, the 70% acetone extracts contained the highest levels of total polyphenols (30.9$\pm$0.6 mg/g for pods; 32.5$\pm$0.5 mg/g for germ and 200.7$\pm$3.0 mg/g for germ with cuticle) and total catechins and procyanidines (6.1$\pm$0.1 mg/g for pods; 6.3$\pm$0.2 mg/g for germ and 49.3$\pm$1.1 mg/g for germ with cuticle). These results indicate that the cuticle of the carob seed contributes to a great extent to the higher free radical scavenging activity observed.

Intracellular toxicity of beta-amyloid: Increase peroxide production in isolated mitochondria

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A central question in Alzheimer's disease concerns the mechanism by which beta-amyloid contributes to neuropathology, and in particular whether intracellular versus extracellular beta-amyloid plays a critical role.

The aim of this study was to determine whether beta-amyloid 1-42 peptide causes cellular toxicity from inside (intracellularly) or from outside (extracellularly). We tested the hypothesis that beta-amyloid peptide causes mitochondrial toxicity and that it increases free radical production by mitochondria.

We isolated hepatic, brain and brain-synaptic mitochondria from rats. Oxidant production by mitochondria were determined by flourimetry. We found that incubation with nanomolar amounts of beta-amyloid peptide significantly increase the mitochondrial oxidant production. This increase is particularly marked in the case of synaptic mitochondria. Although beta peptide increases peroxide production in mitochondria from all tissues studied, this increase was highest in the case of synaptic mitochondria, second in the case of brain mitochondria and lowest in the case of hepatic mitochondria.

We conclude that beta-amyloid peptide, contrary to most reports, is able to cause cytotoxicity from inside the cell, particularly at the mitochondrial level.

Nitric oxide mediates the up-regulation of BCL-2 expression in human endothelial cells exposed to cyclosporin A

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We have previously reported (Longoni et al. 2001, Faseb J. 15, 731-40) that "in vitro" chronic treatment (4-8 days) with therapeutic doses (0.5-2.5 μM) of the immunosuppressant cyclosporin A (CSA) increases Bcl-2 levels in human umbilical vein endothelial cells (HUVEC). In the present work we have investigated the molecular basis of Bcl-2 up-regulation by CSA in HUVEC. By using real-time PCR, we have observed that the increase in Bcl-2 protein induced by CSA correlates with an increase of bcl-2 mRNA. Using confocal microscopy and the nitric oxide-sensitive probe 4,5-diaminofluorescein diacetate (DAF-2 DA), we observed an increase in NO· production in response to therapeutic doses of CSA. Considering that antioxidants prevent Bcl-2 upregulation by CSA, we have then investigated the role of NO· in the upregulation of bcl-2 messenger by CSA. Preincubation with the NO-synthase inhibitor L-NAME reduced bcl-2 messenger up-regulation in response to CSA. On the other hand, the NO-generator DETA-NO upregulates bcl-2 messenger, thus mimicking CSA action. Our data indicate that CSA upregulates bcl-2 messenger and protein via an increase in NO· synthesis.

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Improving the antiradical and biological properties of flavanols by derivatisation: Novel cysteinyl-flavan-3-ol conjugates

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Much attention is currently being paid to the putative benefits of plant polyphenols. In this study, novel bio-based antioxidant compounds obtained by depolymerisation of grape by-product using cysteine as a nucleophile are presented. Chemical studies show that the cysteinyl flavan-3-ol derivatives were potent free radical scavenging agents in the 1,1-diphenyl-2-picrylhydracyl free radical (DPPH) assay. The new conjugates were more efficient than (–)-epicatechin and Trolox. In the same way, EPR experiments evidenced the formation of stable radicals, which lasted for several days only from the derivatised flavanols. We have also performed theoretical calculations and NMR studies to estimate the contribution of the different X-H bonds to the scavenging effect. Through this information, we suggest possible mechanisms for the antiradical action of the conjugates.

In biological studies, the novel cysteinyl derivatives proved to be more efficient than the underivatised (–)-epicatechin as growth inhibitors of human colon carcinoma HT29 cells and human melanoma A375 cells. Cell cycle and apoptosis studies have been conducted. The gallate-containing compound was the most potent antioxidant and growth inhibitor on both cell lines, with influence on the cell cycle. All compounds triggered apoptosis.

Antioxidant enzymes in purified chloroplasts and chromoplasts from pepper fruits

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Given the economic importance of the pepper fruit and the proposed involvement of AOS in the ripening process, we have monitored the changes in antioxidant enzymes in chloroplasts and chromoplasts isolated from commercial green and red pepper (*Capsicum annuum* L. cv. Herminio) fruits. Plants were grown under glasshouse conditions. Chloroplasts and chromoplasts purified from fruit pericarp by differential and Percoll or sucrose density-gradient centrifugation, revealed a high level of intactness and a low degree of contamination by other cellular components. Analysis of the SOD activity pattern by native PAGE showed the presence of four SODs in fruit pericarp, which were identified as Mn-SOD, Fe-SOD, CuZn-SOD I and CuZn-SOD II, whereas in chloroplasts and chromoplasts, only Fe-SOD and CuZn-SOD II were present. In general, total activity values of the enzymes associated with the ASC-GSH cycle (APX, MDHAR, DHAR and GR) were higher in chromoplasts than in chloroplasts, and in both organelles APX activity was the most abundant. However, when the enzyme activities were expressed on a protein basis, only APX and DHAR activities appeared augmented in chromoplasts. These differences in the activity values suggest that APX and DHAR are more correlated than MDHAR and GR with the overall increase in chromoplast protein content during the transition of chloroplasts to chromoplasts. The high APX activity in chromoplasts may have the effect of allowing increased scavenging of H_2O_2 although the same high APX activity would also imply higher ASC consumption. The contrasting pattern of MDHAR and DHAR in chloroplasts and chromoplasts suggests that in the green stage, fruit chloroplast ascorbate regeneration was more correlated with MDHAR activity, while in the chromoplasts from red fruits, ascorbate regeneration was more associated with DHAR activity.

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How PHGPx interacts with itself during sperm maturation

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Phospholipid hydroperoxide glutathione peroxidase (PHGPx; EC 1.11.1.12), is a broad-spectrum thiol-dependent peroxidase, a regulatory factor in various signaling cascades and a structural protein in sperm. Evidence identifying catalytic intermediates of the selenoprotein by LC/ESI-MS/MS is presented. In the ground state enzyme E selenium is present as selenocysteine at position 46. The selenenic acid form, which is considered to be the first catalytic intermediate F formed by reaction with hydroperoxide, could not be identified. The second catalytic intermediate G was detected as Se-glutathionylated enzyme. According to molecular models, specific binding of GSH and of GSSG is inter alia facilitated by electrostatic attraction of Lys-48 and Lys-125. Polymerization of PHGPx is obtained under oxidizing conditions in the absence of low molecular weight thiols. Analysis of MS spectra revealed that the process is due to a selective reaction of Sec-46 with Cys-148' resulting in linear polymers representing dead-end intermediates G'. Also FT docking of PHGPx molecules suggests a reaction of Sec-46 with Cys-148´. It is concluded that the same catalytic principles, depending on conditions, can drive the seemingly diverse actions of PHGPx, i. e. hydroperoxide reduction, GSSG reduction, S-derivatization and self-incorporation into biological structures.

Dietary supplementation of essential fatty acids and oxidative stress in rats

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Introduction. In view of the promising for use of n-3 PUFA in the prevention of chronic diseases, it is necessary to ensure that their consumption does not result in detrimental oxidative effects.

Objective of this study was to compare the extent of oxidative stress elicited in rat hepatocyte by n-6 or n-3 polyunsaturated fatty acids supplementation of diet.

Material and methods. Wistar rats received either 3.3 g sunflower oil/kg of food (SO group) or 7.5 g flaxseed/kg of food (FS group). Phospholipid fatty acid composition was determined by capillary gas chromatography in liver membranes. Liver concentrations of lipid peroxides (monitored as malondialdehyde formation), and liver anti-oxidants such as catalase, superoxide dismutase, glutathione peroxidase and reduced glutathione were measured.

Results. We found a different fatty acid profile in liver membrane between the two rat groups. Sum n-3 was significantly higher in FS group as compared to SO group (2.63$\pm$3.06% vs. 1.3$\pm$1.33%) in liver membrane. Sum PUFA (28.72$\pm$5.42% vs. 23.84$\pm$9.30%), unsaturated index (101.42$\pm$18.16 vs. 78.73$\pm$26.28), and essential fatty acid index (1.63$\pm$0.51 vs. 1.35$\pm$0.78) were significantly higher in SO group. Corresponding to higher essential fatty acid content, malondialdehyde was significantly higher in SO group as compared to FS group (0.15$\pm$0 mmol/mg protein vs. 0.08$\pm$0.01 mmol/mg protein). Elevation of glutathione peroxidase was noticed in enriched linoleic acid diet (1.19$\pm$1.13 mmol/mg protein vs. 0.8$\pm$0.02 mmol/mg protein) and it could be explained as a protective mechanism against the oxidative damage.

Conclusions. Dietary enrichment in flaxseed induced a lower oxidative stress as compared to linoleic acid supplementation.

Characterization of the transcriptional coactivator PGC-1 function in vascular endothelial cells. Protective role from mitochondrial oxidative stress damage

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Chronic hyperglycemia, found in all forms of diabetes plays an important role in the pathogenesis of vascular complications (N ENG J Med, 1993)(Wei et al., 1998). It has been recently found that the vascular damage is a direct consequence of the hyperglycemia-induced overproduction of superoxide in the mitochondria, and the resulting oxidative stress (Brownlee, 2001; Nishikawa et al., 2000).

PGC-1 is a transcriptional coactivator that plays a major role in the regulation of oxidative metabolism, inducing mitochondial activity and proliferation as well as oxidative lipid metabolism, in response to stimuli such as cold, exercise and fasting-re-feeding. Originally described as a thermogenic factor in brown adipose tissue, it was found to be abundant in tissues with high metabolic rates such as muscle, heart, liver and kidney (Reviewed in Knutti and Kralli 2001).

Experiments carried out in human vascular endothelial cells (HUVEC) have suggested that the activation of oxidative metabolism (Hardie and Carling, 1997), prevents the cellular damage associated with hyperglycemia (Ido et al., 2002), while it stimulates lipid metabolism, and suppresses glycolysis (Dagher et al., 2001).

We have determined that PGC-1 is present in vascular endothelial cells where its over-expression induces genes involved in mitochondrial activity, as well as genes that participate in mitochondrial oxidative stress protective machinery. As a consequence of this induction a marked decrease in total cellular ROS levels can be observed in cells that over-express PGC-1.

These results lead us to propose that PGC-1 might be the factor that would coordinate the induction of oxidative metabolism and the expression of mitochondial oxidative stress protective genes, and therefore play a major protective role against oxidative stress of mitochondrial origin.

Time-course of rosmarinic acid production by lavandin (*Lavandula x intermedia*) cell cultures

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Rosmarinic acid (α-*O*-caffeoyl-3,4-dihidroxiphenyllactic acid) is a caffeic acid derivative occurring in several higher plant families, ferns and hornworts. This compound has been shown to display a number of biological activities, including antioxidant, astringent, antiinflammatory, antimutagen, antibacterial and antiviral activities. Furthermore, rosmarinic acid could provide protection against cancer and there is increasing concern about its use as food and cosmetic additive. In vitro production of rosmarinic acid by plant cell cultures has been successfully carried out using several high-producing cell lines. In this work, the accumulation of rosmarinic acid and other related compounds during the culture cycle of lavandin cells is described. Data on antioxidant activity of cell extracts are provided and correlations with the levels of rosmarinic acid found in the different stages of the culture established. Finally, the evolution of enzymatic activities possibly involved in rosmarinic acid metabolism (namely peroxidase, polyphenoloxidase and β-glucosidase) is shown and discussed in relation to the optimization of the production of this compound.

Histological Evidence for enhanced wound healings in diabetic rats supplemented with high dose palm vitamin E

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We have previously shown that palm vitamin E accelerates excision and incision wound closures in diabetic rats when supplemented at a high dose. In order to establish that palm vitamin E improves the rates of wound healing, we examined the histological changes that occur during the process of healing in supplemented and unsupplemented diabetic rats.

Diabetes mellitus was induced using streptozotocin (50 mg/kg bw) intramuscularly in 24 fasting male Sprague Dawley rats. Experiment was started by creating four 6mm diameter wounds on the back of each animal under sterile conditions using a punch biopsy. The rats were then supplemented orally with either palm vitamine E at doses of 5, 50 or 200 mg/kg bw or equivalent volume of olive oil daily. Wounds were collected ten days after the initial injury, cross sections were cut and stained with Haematoxylin's & Eosin stain and Van Giesons stains.

Results showed that rats supplemented with 200 mg/kg bw PV had the most proliferation of cells and growth of collagen fibres indicating improved healing compared to the other groups.

Moderate exercise increases lifespan and cytochrome oxidase activity and decreases oxidative stress in mice

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Mice, 28 wk old, received a moderate training in a treadmill (10, 15 and 20 cm/sec, for 5 min each, every week up to 78 wk of age). Liver, heart, kidney and brain mitochondria were isolated, submitochondrial membranes were prepared, and TBARS, mitochondrial protein carbonyls and the activities of cytochrome oxidase, NADH-cytochrome c reductase and succinate –cytochrome c reductase were determined. Moderate exercise, from 28 to 52 weeks of age: (a) increased mice median lifespan by 19% and 9% in males and females, respectively; (b) decreased mitochondrial TBARS by 10 %, 13 %, 19 % and 20 % in liver, heart, kidney and brain; (c) decreased mitochondrial protein carbonyls by 15 %, 14 %, 12 %, and 20 % in liver, heart, kidney and brain, and (d) increased cytochrome oxidase activity by 6-26 %, 19-25 %, 28-41 %, and 15-20 % in liver, heart, kidney and brain. Cytochrome oxidase activity inversely correlated with mitochondrial protein carbonyls. Other mitochondrial activities were not affected by moderate exercise. It is concluded that moderate exercise at middle age in mammals appears effective in increasing lifespan, likely by a decrease in cellular oxidative stress and by preventing the physiological decline of mitochondrial activities, such as cytochrome oxidase, upon normal aging.

High Doses of Vitamin E Increase Lifespan and Decrease Mitochondrial Oxidation Markers in Mice

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The antioxidant properties of vitamin E are well known and other regulatory functions have been recognized. Vitamin E supplementation of the diet is used to provide a high bioavailability and to assess its biological functions. Diet supplementation with vitamin E and other antioxidants produced variable results in different species and depending on the dose. In this study 160 mice received a high dose of vitamin E in the diet (4.3 g vitamin E/kg food), starting at 48 wk of age and for the rest of their lives. The dose (0.18 mg vitamin E/kJ of basal metabolism) is comparable to a human daily dose of 1200 mg vitamin E/day.

Vitamin E supplementation increased median lifespan by 27% in males (from 65 wk to 83 wk), and by 11% in females (from 84 wk to 93 wk).

Vitamin E supplementation decreased the level of oxidation products in mitochondrial membranes at 78 wk of age. Mitochondrial TBARS in liver, heart, kidney and brain were decreased by 24-39%, 15-19%, 19-23% and 27-32%, respectively, by vitamin E supplementation, considering females and males. Mitochondrial protein carbonyls in liver, heart, kidney and brain were decreased by 17-27%, 19-21%, 23-28%, 17-20%, respectively, by vitamin E supplementation in females and males.

Chronic treatment, from middle age, with high doses of vitamin E resulted effective in increasing mice lifespan, likely by a decrease in mitochondrial and cellular oxidative stress.

Doxorubicin activates nuclear factor-kappaB in primary cultures of rat hepatocytes

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The anthracycline antibiotic doxorubicin is one of the most potent anticancer drugs in clinical use. However, its clinical utility is limited by severe, cumulative and dose-dependent cardiotoxicity. The cytotoxic actions are associated with the generation of free radicals, the induction of membrane alterations through lipid peroxidation and the impairment of DNA and RNA synthesis. Oxidative stress can induce different biological processes, including cell growth, apoptosis and cell adhesion, presumably by stimulating signal transduction and the generation of lipid second messengers, such phosphatidic acid and diacylglycerol (DAG). Previous data have shown that doxorubicin induces the accumulation of [^{3}H]-DAG from [^{3}H]-labeled hepatocytes. In the present work the possible activation of the nuclear factor-kappaB (NF-κB) has been examined in hepatocyte cultures exposed to doxorubicin. NF-κB is composed of transcriptionally active p50/p65 heterodimers. The transcription factor is activated through the proteolytic degradation of IκBα, translocation of NF-κB to the nucleus, and binding to DNA. Western blot analysis with anti-IκBα and anti-p65 antibodies was used. Doxorubicin (10, 20 and 50 μM) induced the cytosolic degradation of IκBα at 3 and 6 h, and concomitantly the NF-κB translocation into the nucleus. This effect was dose-dependent. The possible involvement of free radical in the NF-κB response is also discussed.

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Effect of Long Term Palm Oil Vitamin E Supplementation on Rate of Wound Closure and Lipid Peroxidation at Different Age Groups in Rats

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Vitamin E has been shown to reduce lipid peroxidation which has been reported to increase during aging. It has also been shown to increase rate of wound healing, while aging process has been associated with impairment of the wound healing process. The aim of this study is to determine the effect of long term supplementation of palm oil vitamin E (70% γ-tocotrienol and 30% α-tocopherol) on the rate of wound healing at different age groups and to see any correlation with its oxidative status. Forty male Wistar rats aged three months were divided into two groups. Group A being the control group were given olive oil, while group B were supplemented with palm oil vitamin E at 30mg/kg body weight diluted in olive oil. The wounds were created every six months following supplementation of palm oil vitamin E and the study has been carried out for twelve months. The preliminary results showed that the rate of wound closure was not significantly different in the rats supplemented with palm oil vitamin E as compared to the unsupplemented group in all the groups studied, but there was a significant delay in the rate of wound closure between different age groups in the unsupplemented group but the delay was not significant in the supplemented group. Malondialdehyde (MDA) levels were determined and there was a significant increase in the control group during aging. However in the supplemented group, the MDA levels appeared not to increase. In conclusion, long term supplementation of palm oil vitamin E reduced the delay in wound closure in rats and this appeared to correlate with MDA levels.

In vitro antioxidative properties of reduced nicotinamide adenine dinucleotide

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NADH is a strong reducing agent an has been suggested to act indirectly as an antioxidant. Only recently the activity of NADH as a directly acting antioxidant has been reported and NADH was proposed to be of major importance as antioxidant in mitochondria.

As the data concerning antioxidative activity of compounds strongly depend on the methods used we have investigated antioxidative properties of NADH by different in vitro methods. Scavenging of nitrogen centered radicals as well as superoxide has been determined. Furthermore the influence of NADH on oxidation of low density lipoprotein (LDL) induced by peroxyl radicals was measured. In addition we investigated the action of NADH upon hydroxylation of salicylic acid in the presence or absence of transition metal ions.

We found that NADH acts as scavenger of nitrogen centered radicals like 2,2'-azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS) and diphenylpicrylhydrazyl (DPPH) but not of superoxide. In LDL oxidation we found a dramatic antioxidative effect of NADH, like we found for ascorbic acid. However, the antioxidative effect was lasting longer using NADH compared to ascorbic acid under the same conditions. Ascorbic acid in combination with ferric or cupric ions is a strong hydroxylation agent. In such an assay NADH was found to have only a minor hydroxylating activity. Moreover the stability of NADH is much less influenced by metal ions as it is for ascorbic acid.

We conclude from our results that NADH might be a useful antioxidant with the advantage that it has a much lesser tendency to become prooxidative as does ascorbic acid.

Effect of ferric ions on the antioxidant activity of olive polyphenols

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The effects of five phenolic compounds, occurring in olives and virgin olive oil, namely oleuropein, hydroxytyrosol, hydroxytyrosol acetate and 3,4-dihydroxyphenylethanol-elenolic acid (3,4-DHPEA-EA) and 3,4-dihydroxyphenylethanol-elenolic acid dialdehyde (3,4-DHPEA-EDA), on the oxidative stability of stripped olive oil and stripped olive oil-in-water emulsions were studied in the presence or absence of ferric chloride. The addition of phenolic compounds in bulk oil, even in the presence of ferric ions, significantly extended the induction time of lipid oxidation. The effect of the presence of ferric ions on the antioxidant activity was more severe for compounds with lower antiradical power and more reducing capacity.

In emulsions, oleuropein and hydroxytyrosol enhanced the prooxidant effect of ferric chloride at pH 3.5 and pH 5.5. 3,4-DHPEA-EDA reduced the prooxidant effect of ferric chloride at pH 5.5 and pH 7.4 but at pH 3.5 prooxidant effects were evident at higher phenol concentration. 3,4-DHPEA-EA reduced the prooxidant effect of ferric ions at all pH values tested. In this system, in addition to the antiradical power and reducing capacity, the stability of chelates formed play an important role on the antioxidant capacity of these compounds in the presence of ferric ions.

Radical scavenging activity of dihydroxy- and trihydroxyphenolic acids

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The evaluation of antioxidant *vs.* antiradical activity of phenolic compounds is nowadays an important research area in the field of Food and Medicinal Sciences. Phenolic acids and flavonoids were widely investigated as potentials models for the development of new primary antioxidants, which could prevent or delay *in vitro* and/or *in vivo* oxidation processes.

The phenolic acids used in this study are either of natural or synthetic origin and reveal to be promising anticancer agents [1]. It is worthwhile to refer that some of these compounds were identified as metabolites of flavonoids which reinforces the importance of this research.

To get an overview of the antioxidant activity, the preliminary *in vitro* screening of antiradical activity of hydrocaffeic acid, 3,4-dihydroxyphenylacetic acid, protocatechuic acid, and its trihydroxylated counterparts was performed by total antioxidant assays (TAA). The results of this work will be presented.

[1] **"**Anticancer Activity of Phenolic Acids of Natural or Synthetic Origin: a Structure-Activity Study", C.A. Gomes, T. Girão, J.L. Andrade, N. Milhazes, F. Borges, M.P.M. Marques, in preparation.

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Antioxidant defense enzyme activities in the interscapular brown adipose tissue of rats treated with cadmium and coenzyme Q_{10}

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In our study we investigated the effects of exogenous cadmium (Cd, 17 mg/day/kg b.m. in drinking water), coenzyme Q_{10} (CoQ_{10}, 16 mg/kg/dose CoQ_{10} dissolved in olive oil, i.m., every fifth day) and Cd+CoQ_{10} (in above mentioned amounts) on antioxidant defense enzyme activities (Total SOD, Mn SOD, CuZn SOD, CAT, GSH-Px, GST and GR) in interscapular brown adipose tissue (IBAT) of male two months old *Wistar albino* rats during 30 days.

Cd induces significantly increased Mn SOD activity, while concomitant treatment of animals with Cd+CoQ_{10} reversed this change. The activity of CuZn SOD was significantly decreased both in Cd and Cd+CoQ_{10} treated animals. CAT activity was significantly increased in rats treated with Cd, whereas Cd+CoQ_{10} normalized the activity of this enzyme. The activity of GSH-Px was significantly increased in all investigated groups of animals. Cd induces an increased activity of GST, but by concomitant treatment of rats with Cd+CoQ_{10} the GST activity was retained. The activity of GR was significantly increased in Cd treated animals, while in rats treated with Cd+CoQ_{10} was significantly decreased.

It can be concluded that Cd induces oxidative stress and alter the activities of some antioxidant defense enzymes in IBAT of rats. It is also shown that CoQ_{10} can normalized Mn SOD, CAT and GST activities after Cd-induced changes.

Enzymatic reduction of glutathione by dihydrolipoamide: characterization of a new activity of glutaredoxin

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Glutaredoxins (Grx) are small proteins which catalyze thiol disulfide oxidoreductions involving glutathione (GSH) and disulfides in proteins or small molecules. In a previous report [1], we have shown the ability of glutaredoxins to catalyze the reduction of oxidized glutathione (GSSG) by dihydrolipoamide (DHL), an important biological redox catalyst and synthetic antioxidant. We have characterizated this activity with regard to specificity, kinetic and physico-chemical parameters. Glutaredoxin catalyzes specifically the reduction of GSSG by DHL. So, other disulfides such as hydroxyethyldisulfide (HED), cystine instead of GSSG did not react. Thioredoxin could not catalyze the reduction of oxidized glutathione or other disulfides (cystine, insulin) by dihydrolipoamide. Kinetic parameters (Km and Kcat) have been determined for recombinant glutaredoxins. The Km values for GSSG fluctuated between 0.24 mM for human Grx and 0.92 mM for yeast Grx2. For DHL, the Km values varied between 0.15 mM for *E. coli* Grx2 and 0.29 mM. DHL-dependent activity was highest with *E. coli*Grx2 (kcat=3.235 min^{-1}) and lowest with human mitochondrial Grx2a (kcat=96 min^{-1}). The optimal pH for this new activity of glutaredoxin is 8.0, compared to pH 9.0 for the standard HED reduction activity.

[1] Porras P., Pedrajas J.R., Martínez-Galisteo E., Padilla C.A., Johanson C., Holmgren A. & Bárcena J.A. BBRC (2002) 295 1046-1051.

Blockade of MAP kinase activation in acute pancreatitis by simultaneous inhibition of TNF-α production and xanthine oxidase

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Background: Pancreatic injury triggers two major pathways related to MAP (mitogen activated protein) kinases and involved in the systemic effects of severe acute pancreatitis: proinflamma-tory cytokines and oxidative stress. Aim: To assess the effects of inhib-iting both TNF-α production and xanthine oxidase activity on the inflammatory response, MAP kinase activation as well as glu-tathione depletion and oxidation in necrotizing acute pancreatitis in rats.

Methods: Pancreatitis was induced by intraductal infusion of 3.5% sodium taurocholate. We examined whether treatment with oxypurinol – a specific inhibitor of xanthine oxidase- and/or pen-toxifylline – an inhibitor of TNF-α production- affects pancreatic damage, glutathione depletion and oxidation and MAP kinase phosphorylation in pancreas.

Results: Acute pancreatitis induces phosphorylation of all three Map kinase families (p38, JNK, erk 1/2) in pancreas 30 minutes after pancreatitis induction and decrease progressively as the pan-creatitis goes on. Oxypurinol prevented glutathione oxida-tion as well as p38 phosphorylation. Pentoxifylline prevented glutathione depletion and erk 1/2 and JNK phosphorylation. Combined treat-ment with oxypurinol and pentoxifylline almost completely abol-ished glutathione depletion, glutathione oxida-tion and MAP kinase phosphorylation in pancreas. Histology revealed a reduction in pancreatic damage.

Conclusions: Simul-taneous inhibition of TNF-α production and xanthine oxidase activity greatly reduced local inflammatory response in acute pancreatitis. This effect was associated with blockade of the three major MAP kinases.

Biomarker responses in the clam, *Scrobicularia plana,* in relation to a sewage treatment plant (STP) in the Cádiz bay

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Bivalves are organisms currently used as bioindicators for pollution monitoring. One of the reasons for this choice is their sessile habits and low metabolism, which makes them suitable for determining the presence of certain pollutants in selected habitats. Despite their low metabolism they respond to the presence of certain chemicals, as higher organisms do, although to a much lesser extent.

In the present work we selected the native clam *Scrobicularia plana* in four sites, one situated at the mouth of the Río San Pedro, and considered as control, and the other three in the Caño Sancti-Petri area (Cádiz, NW Spain) at an increasing distance from the sea influence. The aim was to relate the biological responses on this species to the pollution gradient. The gradient was caused by the release of the domestic and wastewater from the city of San Fernando. This release ceased in January 2002 with the construction of a sewage treatment plant (STP), and sampling of the clams was undertaken in June 2002, six months later.

The biomarkers of organic pollution selected were the antioxidant enzyme catalase (CAT), the cytochrome P450 dependent ethoxyresorufin *O*-deethylase (EROD) activity, the phase II detoxifying enzyme glutathion S-transferase (GST) and the neurotoxicity marker acetylcholinesterase (AChE). All the enzymes were measures in the S12 fraction of the digestive gland and 6 replicates per assay were made from a pool of 3 individuals each. CAT and GST did not show any trend, EROD activity increased with increasing sea distance although statistical analysis of the data using the ANOVA test did no reach significance. In contrast AChE was significantly depleted in all sites in the Caño Sancti-Petri in relation to the control site.

These results do not strongly indicate biological effects of a domestic waste-water pollution gradient in a sedentary organism in most of the biomarkers selected, however determinations in the S12 homogenate may not be the best fraction to determine these activities, at least as far as CAT, EROD and GST are concerned.

Influence of normal and controlled atmosphere on quality of three clones of pear (*CV. ROCHA*)

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"Pêra-Rocha" is the most important pear (*Pyrus communis* L.) cultivar grown in Portugal. Several clones of this *cv*. have been selected and their productivity characterized. Clones behaviour after harvest and during storage remains unknown. The goal of the present study was to identify the pear clone that maintains the best quality after a long period of storage.

Three clones of pear *cv*. *Rocha* harvested at 29/08/01 (co mmercial harvest) were maintained at normal (0ºC temperature; 90-95% HR) and controlled atmosphere (0.8ºC temperature; 90-95% HR; 3% O_2; 2% CO_2) for six months.

At the end of storage and the subsequent shelf life period at 20ºC (1 week) several physiological parameters were analysed: (total protein, solute leakage, lipoperoxidation) and changes on activity of antioxidant enzymes such as superoxide dismutase, ascorbate peroxidase, glutathione reductase and catalase.

Results suggest that at the end of storage fruits under controlled atmosphere have a higher quality than those under normal atmosphere. Clone 2 seems to be the most promising.

The present study showed, that in general, controlled atmosphere provides a beneficial effect on the quality of the pears after a long storage period, delaying the senescence process.

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Analysis of Aminochromes by HPLC-Photodiode Array. Adrenochrome Evaluation in Rat Blood

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The catecholamines oxidation process and its products may induce cardiotoxicity (Remião et al., 2002) and neurotoxicity (Halliwell and Gutteridge, 1999). Catecholamines can oxidise to aminochromes through autoxidation or by enzymatic or non-enzymatic catalysis. Although some toxic effects seem to be related with the formation of aminochromes there is still scarce information concerning the identification and evaluation of these reactive compounds in *in vivo* models.

In this study five catecholamines were oxidised to their respective aminochromes: adrenaline to adrenochrome, noradrenaline to noradrenochrome, dopa to dopachrome, dopamine to dopaminochrome and isoproterenol to isoprenochrome. An isocratic reverse phase HPLC-Photodiode Array detection method was developed to analyse each pair catecholamine/aminochrome. The analytical system was then applied to the detection of adrenochrome in rat blood at 490 nm. Thus, adrenochrome was administered i.p. to rats and its concentration in whole blood was monitored after 5, 15 and 25 minutes. Blood treatment for adrenochrome evaluation consists of an acidification for protein precipitation followed by a rapid neutralization. The results showed a rapid decrease of adrenochrome concentration in blood after its administration. The adrenochrome present in blood was characterised by UV and tandem mass spectrometry.

Thus, the development of this methodology allows a direct quantification of aminochromes in blood or tissues and the correlation of its levels with the observed toxic effects (Remião et al., 2003).

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Applications of flow cytometry in the study of mitochondrial ageing

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Flow cytometers assay particles in suspension which interfere individually with a beam of light. The intersection of each particle with a laser beam causes the emission of a series of light signals which allow us to separate populations in a given sample by its size, or by its reactivity with various fluorochromes.

In our studies mainly mitochondria isolated from Wistar rats young (3 months old), old (24 months) and old treated with anti-oxidants. Antioxidants, usually *Ginkgo biloba*, were given in drinking water at a dose of 100 mg per kilo of body weight for 3 months.

We have used flow cytometry to study the function of isolated mitochondria from brain and the changes in the process of ageing, both at morphological and functional levels.

The following parameters have been measured: mitochondrial membrane potential (Rh123), peroxide levels (Dh123), size, complexity and mitochondrial mass.

We find that ageing causes a decrease in mitochondrial membrane potential and a concomitant increase in the rate of oxidant production. Morphologically, we have found two populations of mitochondria of different size and complexity. In old rats, mega-mitochondria are found. No changes in total mitochondrial mass were found in ageing. All these changes can be diverted by administration of EGb761 extract from *Ginkgo biloba.*

Catechol-*O*-methyltransferase val108/158met polymorphism in women under an *in vitro* fertilization program.

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Catechol-*O*-methyltransferase (COMT) catalizes the *O*-methylation of catechols by transferring the methyl group of S-adenosil-L-methionine to one of the hydroxyl groups of the catechol substrate. The enzyme, thus, prevents the redox cycling of catechols and the subsequent generation of free radicals. These reactive species have been proposed to be involved in the development of certain hormone dependent cancers and estrogen toxicity. The COMT gene codes for both soluble (S-COMT) and membrane-bound (MB-COMT) proteins, the expression of the COMT gene being controlled by two distinct promoters. The level of COMT enzyme activity is genetically polymorphic with a trimodal distribution of low ($COMT^{LL}$), intermediate ($COMT^{LH}$), and high ($COMT^{HH}$) activities. Low COMT activity is associated with enzyme thermolabilty. The molecular basis of the thermolabilty is the substitution of Val108 by Met108 in the S-COMT (or the corresponding aminoacids 158 in the MB-COMT) caused by transition of guanine to adenine at *codon 158* of the COMT gene. In the present work, the COMT val108/158met polymorphism is analyzed by ARMS (*Amplification Refractory Mutation System*) in a population of women enrolling in an *in vitro* fertilization program. The genotype frequency distribution was 26.2% (HH), 21.5% (LL), and 52.3% (LH) for a total of 172 women. This distribution is not significantly different from that reported in the literature for Caucasian populations.

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Key role of xanthine oxidase in weaned induce apoptosis in rat mammary gland

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Although apoptosis is a well defined morphological process, the biochemical mechanisms involved remain under investigation. It is well known that cellular redox status modulates various aspects of cellular function. Several reports have emphasized the role of oxidative stress and nuclear DNA damage in apoptosis. Here we show the role of xanthine oxidase (XO) in the maintenance of apoptosis. 3 month old Wistar rats were used. Lactating mammary glands were from control, 12, 24, 48 hour weanned rats treated or untreated with 120-150mg allopurinol p.o. XO activity was measured by fluorimetry. We measured GSH and GSSG levels in mammary gland homogenates using a HPLC method with UV-V detection. We use Gong protocol to detect digested DNA from apoptotic mammary gland tissue and NeuroTACS kit for the morphological apoptotic determination. DNA digestion takes place at 12h after weaning in rat mammary gland, during involution. Glutathione oxidation precedes nuclear DNA degradation. There is a significant increase in XO activity 24 h after weaning. The increase in XO activity could be due to an increased proteolytic activity in the mammary gland. MAPK p38 a stress activated proteine kinase is activated by phosphorylation. This kinase increase at 48h after weaning demonstrate the implication in apoptosis. P53 level increase at 24h after weaning. Allopurinol administration, decreased significantly XO activity. This inhibition induces a decrease in apoptosis. Thus XO is necessary in order to maintain high ROS levels during the apoptotic process. Although XO does not trigger apoptosis, its role in the maintenance of the apoptotic machine appears very important .

Antioxidative enzymes expression and signal transduction in pea plants under cadmium toxicity

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Cadmium is a toxic trace pollutant for humans, animals and plants. In previous works, evidence were obtained showing that Cd can induce oxidative stress by diminishing antioxidant defences or increasing ROS production [1]. In this work, using pea plants, the effect of 50 μM $CdCl_2$ on the transcript levels of different antioxidative enzymes was studied. The mechanisms involved in cell response against Cd were also investigated by using signal transduction modulators, as well as the possible involvement of programmed cell death (PCD) in the heavy-metal toxicity.

In leaves, cadmium produced changes in the glutathione reductase expression at transcriptional level, and in the expression of catalase and CuZn-SOD at translational and posttranslational level. However, the expression of ascorbate peroxidase, Mn-SOD and monodehydroascorbate reductase was not modified by Cd. The regulation of those antioxidants was partly dependent on protein dephosphorylation, Ca^{2+} concentration, $NO^{\cdot}$, salicylic acid, cGMP, and ROS. These results evidence a similarity between the response to Cd toxicity and the hypersensitive response to pathogen infection, although in pea leaves PCD was not induced by Cd.

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Formation of free radicals in the stimulation of the mitochondrial permeability transition by dihydrolipoate

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Dihydrolipoate (DHLA) stimulated superoxide (O_2^-) formation and the mitochondrial permeability transition (MPT) in rat liver mitochondria (1999 OCC p. 218). With pyruvate as substrate the stimulation was seen with the lowest [DHLA], <10 μM, see Morkunaite *et al.* Biochem Pharmacol 2003;65:43. TEMPO (O_2^- scavenger) and BHT (lipid radical scavenger) prevented this, but not desferrioxamine. The prooxidant effects of DHLA were thus not due to its Fe complex in the medium or contaminating lysosomes but could be due to interaction with desferrioxamine-inaccessible redox centers in mitochondria. Stimulation of O_2^- generation by DHLA was confirmed in heart submitochondrial particles respiring on succinate. Thiyl radicals may be formed by one-electron oxidation of DHLA by free radicals such as ubisemiquinone. DHLA increased the rate of O_2 consumption in antimycin A or myxothiazol-treated mitochondria (but not in the presence of both compounds), which may be due to O_2^- production from O_2 or to reduction of cyt c and its subsequent oxidation by Complex IV. DHLA inhibited State 2 and uncoupled respiration on NAD^+-dependent substrates by induction of MPT, and possibly also by inhibition of pyruvate and α-ketoglutarate dehydrogenase by peroxidation products formed. Succinate respiration was also inhibited after a lag.

Hydrogen peroxide decomposition in *Amphiprora Kufferathii* supported by epiphytic bacteria

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Microalgae that colonize the surface, interior, and bottom of sea ice, as well as the under-ice platelet layer are called ice algae. Bottom ice algae, dominated by diatoms, are commonly found in diffuse lower layers of congelation ice as well as in the lower platelet ice layer in some parts of the Antarctic.

An abundant and diverse bacterial community exists within brine channels of annual sea ice and at ice sea water interface. 93% of the bacterial biomass are located in the bottom 20 cm of sea ice.

Epibacteria appeared to be selective in the colonisation of diatoms, as they are found predominantly on healthy *Amphiprora*-cells. We earlier showed that the algal cells as well as the epiphytic bacteria contain catalase, the hydrogen peroxide degrading enzyme. Antarctic sea ice normally contains concentrations of hydrogen peroxide up to 500 nmol * L^{-1}, dependent on effects of light intensity and temperature.

We hypothesize that the epibacteria are involved in the enzymatic antioxidative defence of the *Amphiprora*-cells.

The role of oxidative stress and antioxidant treatment in a model of type 2 diabetes

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One of the possible factors playing an important role in the pathogenesis of diabetes and its vascular complications are free radical reactions induced by reactive oxygen species. It has been extensively reported that antioxidant substances may be helpful for the prevention and treatment of diabetic complications. The importance of antioxidant supplementation for diabetics remains an unresolved issue The present study was undertaken to examine the effects of soybean oil, alpha-tocopherol and coenzyme Q_{10} (CoQ_{10}) on metabolic control and on the pancreatic mitochondria of Goto-kakizaki (GK) rats, a model of type 2 diabetes. Soybean oil, alpha-tocopherol and CoQ_{10} were injected during 8 weeks in diabetic rats. Fasting plasma glucose and HbA1c were significantly decreased in the different groups treated with antioxidants. There was no variation in intraperitoneal glucose tolerance test. The levels of the lipophilic antioxidants, coenzyme Q and alpha-tocopherol were evaluated in the plasma and pancreatic mitochondria. Diabetes induced a decrease in coenzyme Q plasma levels that prevailed after treatment with antioxidants. Moreover, the plasma α-tocopherol levels were higher after treatment with the antioxidants. An increased content of both antioxidants was observed in pancreatic mitochondria of treated GK rats. In conclusion, our observations indicate that antioxidant treatments may be beneficial effects in type 2 diabetes.

Determination of Antioxidant Activity (AOA) in Human Saliva, using the Koracevic Method in the Presence of Artificially Added Antioxidants.

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The saliva antioxidant pool includes mainly peroxidases and uric acid (UA), but ascorbic acid (AA), albumin (Alb), superoxide dismutase, and some vitamins also contribute to the antioxidant defense mechanism. The Koracevic method (2001) was used to determine the antioxidant capacity in human saliva and the sensitivity of this method in the presence of artificially added antioxidant (ADD) substances (UA, AA, Alb, Trolox). Four saliva samples from a healthy female subject were collected, each on a different day. The Koracevic method was applied to 50 µL of saliva (control) and 50 µL of saliva spiked with ADD (UA = 0.05 mM, Alb = 0.01 mg/µL, AA_1 = 0.05 mM, AA_2 = 0.50 mM, Trolox (Tro) = 0.05 mM), each sample was measured by triplicate, the means of these sets were used to calculate the AOA for each assay. The values of AOA (mM, as UA) for the control and experimental samples respectively, were: UA = 0.938 and 0.951; Alb = 0.831 and 0.826; AA_1 = 0.991 and 0.915; AA_2 = 0.991 and 0.850; Tro = 0.883 and 0.933. The method is reproducible and efficient. It can detect the prooxidant effect of AA, however, it seems to be moderately sensitive in the presence of artificially added antioxidant substances in the used concentrations. In this work the saliva antioxidant capacity is revealed to be very effective, by the fact that the concentration of ADDs must be relatively high in order to multiply the antioxidant action of the human first barrier, the saliva.

Progress towards the discovery of the pharmacophore of hydroxycinnamic acids as a new class of primary antioxidants

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Although there is general agreement that catecholic compounds possess radical scavenging properties its mechanism of action is not fully understood so far. It is usually assumed that the radical scavenging activity is related with its hydrogen or electron donating ability, and/or with the stability of the resulting phenoxyl radicals.

Cinnamic acids (β-phenylpropenoic acid) are extensively investigated as potential models for the design and development of new primary antioxidants. Preliminary structure-activity relationship (SAR) studies performed with hydroxycinnamic derivatives have shown that an *ortho*-dihydroxyl moiety is essential for free radical scavenging activity, even though the role of the conjugated double bond Cα=Cβ in the activity is still controversial. To get an insight of the SAR of this type of compounds new analogs were synthesised: 2,3-dihydroxycinnamic acid and 3-(2,3-dihydroxyphenyl)propanoic acid. The synthetic strategy and structural data of the compounds will be presented. The antioxidant profile of the phenolic acids is being evaluated and compared with that shown by caffeic and hydrocaffeic acids.

Structure-property studies on the antioxidant activity of flavonoids present in grapes and wine

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The screening of flavonoids from natural sources for their bioactivities as antioxidants is usually assessed by evaluation of its profile as chain-breaking antioxidants, *i.e.* through the measurement of free radical scavenging activity. As other mechanisms may underlie the activity it is found to be important to check, for instance, the ability of phenolic compounds to chelate transition metal ions. With this purpose, studies were carried out with three flavonoids (quercetin, taxifolin, catechin), being the acidity and the formation constants of copper II/flavonoid systems performed by potentiometric and spectrophotometric automatic titrations. On the other hand, in order to achieve structure-property-activity relationship, it is essential to acquire other physicochemical parameters such us the partition coefficient (Kp), which allow to check the distribution of the antioxidants between two distinct environments in biological systems and thereby the degree of their interaction with the membrane phospholipids. The quantification of this behaviour can be done in micellar media which is classified as a biomimetic membrane model. In the present work the results of Kp evaluation of the three flavonoids determined by derivative spectrophotometry in neutral, positive and negative charged micelles will be also presented.

Oxidation derived metabolites of β-carotene are able to initiate apoptosis in S-Type SHEP neuroblastoma cells

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β-Carotene was reported to exert anticarcinogenic effects. As a result the substance is widely used as prophylactic agent in food and medicine. However, there are still controversies about its potential toxicity after few clinical efficacy trials failed. Moreover, there is some evidence that β-carotene cleavage products (CP) may exert harmful effects in smokers. To investigate those effects, β-carotene was oxidized by HOCl/⁻OCl to form CP mixtures for further experiments. Several apo-carotenals, retinal, β-ionone, but also a few short-chain epoxides were identified by HPLC and GCMS as metabolites in these mixtures. In the present work the potential of β-carotene CP to initiate apoptosis was studied in S-type SHEP neuroblastoma cells. In the experimental settings different mixtures of β-carotene CP but also CP available as single compounds were tested. The results indicate that incubation with mixtures containing long chain compounds including retinal and incubation with retinal as single compound in concentration ranges of 10–50 µM leads to apoptosis in S-type SHEP neuroblastoma cells. It should be noted that the β-carotene CP in the tested concentration ranges were not able to inhibit apoptosis induced by retinal and its metabolites in these cells. Since apoptosis is one of the major anti-cancer mechanisms of the human body these results might be of relevance in the discussion about the negative effects of high dose β-carotene supplementation seen in smokers.

Formation of β-carotene cleavage products after oxidation by hypochlorite – a model for degradation by polymorphonuclear leukocytes?

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β-Carotene, used as supplement in food and medicine, failed in several clinical efficacy trials. Now, there is some evidence that β-carotene in high dosages might be harmful, especially to smokers. These negative effects might be mediated also by carotenoid cleavage products (CP) having a high reactivity towards biomolecules. In previous studies we could show that mixtures of β-carotene CP led to rapid loss of Na^+-K^+-ATPase activity [Siems et al., Free Radic. Res., 2000] and to an impairment of ADP-stimulated respiration in rat liver mitochondria [Siems et al., FASEB J., 2002]. Although a number of β-carotene CP were already identified the mechanism of their formation *in vivo* is still unknown and the product pattern has to be completed. One possibility of oxidative non-enzymatic cleavage of β-carotene in living organisms is HOCl/$^-$OCl produced by polymorphonuclear leukocytes (PML). In *in vitro* experiments stimulated PML in culture were able to cleave β-carotene and lycopene present in the media in physiologically relevant concentrations. To study the mechanisms in detail β-carotene was oxidized in a lipoprotein matrix by added HOCl/$^-$OCl. The experiments were varied by different conditions regarding pH and concentration of lipoproteins. A number of long-chain apocarotenals but also a number of short chain products was identified. The knowledge on product pattern and product formation is essential for the establishment of safe conditions for carotenoid supplementation in disease prevention and clinical therapy.

The Uptake of Tocopherols by RAW 264.7 Macrophages

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Background: The dietary intake of gamma-Tocopherol (gamma-TH) is generally higher than that of alpha-tocopherol (alpha-TH). However, alpha-TH plasma levels are about four fold higher than those of gamma-TH. A preferential cellular uptake of gamma-TH over alpha-TH could contribute to the observed higher plasma alpha-TH levels. We, therefore, studied the uptake and depletion of both alpha-TH and gamma-TH (separately and together) in cultured RAW 264.7 macrophages. Similar studies were performed with alpha-tocopheryl quinone and gamma-tocopheryl quinone, which are oxidation products of tocopherols. Results: RAW 264.7 macrophages showed a greater uptake of gamma-TH compared to alpha-TH. Surprisingly, we also found that the presence of gamma-TH promoted the cellular uptake of alpha-TH. Mass balance considerations suggest that products other than quinone were formed during the incubation of tocopherols with macrophages. Conclusion: Our data suggests that gamma-TH could play a significant role in modulating intracellular antioxidant defense mechanisms. If these results could be extrapolated to in vivo conditions they suggest that gamma-TH is selectively taken up by cells and removed from plasma more rapidly than alpha-TH. This could, in part, contribute to the selective maintenance of alpha-TH in plasma compared to gamma-TH.

Vitamin C interacts with nitric oxide production during apnea diving session

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Nitric oxide plays an important role in the regulation of the neutrophil function. NO is an endogenous modulator of leukocyte rolling and adhesion, degranulation and free radical generation. NO inhibit these functions and could act restricting the inflammatory processes. Some evidences indicate that hypoxia-reoxigenation induces the NO synthesis in neutrophils. The aim of our investigation was to study the effects of apnea diving session and vitamin C diet supplementation on the nitric oxide generation in neutrophils in elite apnea divers. One group was supplemented with vitamin C for a week, and the second was the control group that took a placebo. Venous blood samples were collected before and immediately after apnea diving session, and other blood sampled were collected after one hour of recovery. We determined the activity of arginase, the protein levels of inducible nitric oxide, and the nitrite levels in neutrophils. We also determined the levels of ascorbate in plasma and in neutrophils. Neutrophil arginase activity increased after diving session both in the placebo and in the supplemented group, but this increase was higher in the supplemented than in the placebo. The iNOS levels decreased after diving session in the supplemented group but placebo maintained the basal levels. Neutrophils nitrite levels also decreased only in the supplemented group after diving session. This results suggest that vitamin C affects the neutrophil NO synthesis, affecting to the iNOS levels and the arginine availability, with low nitrite production after apnea diving.

Acute phase immune response to exercise decreased neutrophils antioxidant enzyme defences but unaffected the protein oxidation

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Long-duration or damaging exercise initiates reactions that resemble the acute phase response to infection and induces neutrophils priming for oxidative activity. When neutrophils are activated release granule constituents, which participate in the inflammatory response. The activated neutrophil presents increased capabilities to synthesize reactive oxygen species. However, we evidence a parallel decrease in antioxidant enzymes in neutrophils. Our objective was to bring evidences of this situation, and to determine if the neutrophils release antioxidant enzymes to the extracellullar. We studied the espace the oxidative stress effects of a cycling mountain stage. Ten voluntary male professional cyclists participated in this study. We determined the activities and protein levels of catalase and the two isoforms of superoxide dismutase in neutrophils and plasma, the myeloperoxidase activity in neutrophils, the carbonyl derivates in neutrophils, and the GSH/GSSG ratio. The cycling stage decreased GSH/GSSG ratio, enzyme activities and protein levels of catalase and SOD in neutrophils and increased SOD activity and MnSOD levels in plasma. The MPO activity increased in neutrophils after the exercise. The carbonyl levels didn't change after exercise. Neutrophils could contribute to plasma antioxidant enzyme levels during the acute phase immune response to exercise, and this contribution didn't produce oxidative damage to neutrophils.

MXXCW motif as a starter of protein phosphorylation-linked cellular signalling

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We studied the mechanism of redox reaction-mediated regulation of protein tyrosine kinases (PTKs), which involves oxidative modification of PTK molecules. First, we found that oxidative modification of Src kinase in vitro through sulfhydryl group-reactive Hg or nitric oxide promoted the kinase activity in a Tyr527-independent but Tyr416-dependent manner. Next, we found that ultraviolet irradiation of cells bearing c-RET or extracellular domain-deleted oncogenic RET-PTC-1 promoted RET kinase activity in close association with enhancement of S-S-bonded dimer formation of RET proteins in cells. Study using cells transfected with c-RET (RET-PTC-1) with a Cys987 (Cys376)-> Ala mutation showed that both background catalytic activity and dimer formation absolutely require Cys987 (Cys376). This observation was confirmed by results of experiments using cells with RET-PTC-1 with a Cys376 -> Gly or Cys376 -> Lys mutation. Correspondingly, cell-transforming activity of oncogenic RET-PTC-1 was totally abolished. The magnitude of decrease in the level of in vitro kinase activity of RET-PTC-1 with the cysteine mutation was much more extensive than that of kinase with a Tyr -> Fhe mutation at the major autophosphorylation site. Trans-phosphoryaltion of mutant RET by exgeneously added v-Src, which restored the decreased catalytic activity of RET with a mutation at Tyr981, Tyr952 or Tyr928, did not rescue the activity. Cys987 (Cys376) resides in the MXXCW motif, which was found by a database search to be in 81 out of 83 PTKs. Based on these observations, we propose that this motif fundamentally works as a starter of the kinase activity of PTKs and the downstream serine/threonine kinases.

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Relationships between antioxidants and leaf senescence in nodulated pea plants

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Leaf senescence and associated changes in antioxidants were monitored in commercial pea (*Pisum sativum* L., cv. Phoenix) plants grown under different nitrogen regimes. One group of plants (nodulated) was inoculated with *Rhizobium leguminosarum* and grown with Hoagland's solution without nitrogen. A second group was not inoculated (controls) and these were grown on complete Hoagland's solution containing nitrogen. Plants were grown for 12 weeks until both nodules and leaves had fully senesced. In pea leaves different antioxidants were determined in crude extracts and isolated peroxisomes and mitochondria. Leaf ascorbate contents decreased after 7 weeks in both sets of plants and preceded leaf senescence. The leaf glutathione pool, although declined sharply after 9 weeks, was relatively constant thereafter. In crude extracts, the activities of all antioxidant enzymes decreased after 9 weeks mainly in the nodulated plants. The extent of lipid peroxidation and the number of carbonyl groups was higher in the senescent leaves of nodulated plants. In leaf peroxisomes isolated from senescent nodulated plants, the malate synthase activity, a marker of senescence, was greatly increased but the activities of catalase and hydroxypyruvate reductase were lower compared to controls. Taken together, these results suggest that the leaves senesce earlier on nodulated plants than on nitrogen-fed controls. Leaf senescence is associated with loss of ascorbate and enhanced cellular oxidative damage that is exacerbated in nodulated plants.

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Comparison of Antioxidant and Scavenging Activity of Aloe-emodin, Emodin and Rhein on Free Radical and ROS

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Anthraquinones and dianthrones (1,8-dihydroxyanthra-quinones, DHAs) are present in several plants mainly as glycosides. These compounds have both therapeutic and cytotoxic properties related with diverse types of human affections. For example, aloe-emodin (1,8-dihydroxy-3-hydroxy-methylanthraquinone) is a compound present in *Aloe vera* leaves, which exhibits antifungal, mutagenic, and tumorigenic properties, although in a recent report aloe-emodin was postulated as a new lead antitumor drug; emodin (1,3,8-trihydroxy-6-methylanthraquinone) possesses anticancer, diuretic, antibacterial, vasorelaxant and anti-inflammatory effects, and rhein (1,8-dihydroxyanthraquinone-3-carboxilic acid) is the active metabolite of diacerein, a drug for the treatment of patients with osteoarthritis. In this work we investigated the ability of emodin (**1**), aloe-emodin (**2**) and rhein (**3**) to inhibit free radical or reactive oxygen species ($^{\cdot}OH$, $^1O_2$, H_2O_2) generated in cell-free systems using isoluminol and luminol-enhanced chemiluminescence and electronic absorption spectra. In the presence of **1**, **2** and **3** a dose-dependent inhibition period was seen in this system as assayed by isoluminol-enhanced chemiluminescence (ILCL) with horseradish peroxidase (HRP), as also luminol-enhanced chemiluminescence (LCL) with H_2O_2 or ferrous iron. On the other hand, these hydroxyanthraquinone were capable to quenching the absorbance of ethanolic solutions of galvanoxyl radical. In a separate experiment we observed the trapping of singlet oxygen ($^1O_2$) generated by rose bengal, by the presence of **1**, **2** or **3**. These results suggest that emodin, aloe-emodin and rhein scavenges reactive oxygen and free radicals species in the following decreasing order: emodin > rhein > aloe-emodin.

Antioxidant activity in extracts from coriander

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Since ancient times, spices have been added to different types of food to improve the flavour, and at the same time spices often help to preserve the food. Many herbs and spices are known as excellent sources of natural antioxidants, and may help protect the body against damage by reactive oxygen species. The present study was performed in order to investigate the antioxidant and radical scavenging activity in coriander (*Coriandrum sativum*) and coriander oil. Coriander seeds and fresh leaves of coriander were extracted with solvents of different polarity. The radical scavenger activity of the extracts and coriander oil was evaluated toward 1,1-diphenyl-2-picryl-hydrazyl (DPPH), inhibitory activity toward soybean 15-lipoxygenase and Fe^{2+}-induced peroxidation of porcine brain phospholipids was investigated as well. Our results indicate the presence of more potent antioxidants in the leaves of coriander rather than in the seeds. It also seems that the most potent antioxidants are distributed in the ethyl acetate extract, both for seeds and leaves of coriander. Coriander oil is ineffective as a DPPH scavenger, but has moderate inhibitory activity towards 15-lipoxygenase.

In conclusion, addition of coriander in food may rise the total content of antioxidants, and it seems that leaves of coriander is a better source of antioxidants than coriander seeds.

Oxidized eicosanoids in UV-irradiated human skin and HaCaT-cultures after administration of anti-inflammatory drugs using microdialysis technique

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UV-irradiation of the human skin leads to the induction of oxidative stress and inflammation mediated by reactive oxygen radicals, lipid peroxidation, liberation of arachidonic acid from membrane phospholipids and formation of prostaglandins and leucotrienes. It is the aim of our studies to analyse the levels of oxidized eicosanoids, such as 8-iso-$PGF_{2\alpha}$, $PGF_{2\_}$ PGE_2, monohydroxyeicosatetraenoic acids (HETEs) and LTB_4 in the dermal interstitial fluid obtained by cutaneous microdialysis technique and for comparison in cultured keratinocytes (HaCaTs) after UV-irradiation and application of diclofenac, a nonsteroidal anti-inflammatory drug. Defined areas on the volar forearm of 10 healthy volunteers were exposed to UVB irradiation (20-60 mJ/cm^2). After selected time intervals (starting with 3 and 24 hours), microdialysis membranes were cutaneously inserted beneath the irradiated area and diclofenac was administered topically. The membranes were perfused with isotonic saline solution and microdialysate samples were collected in Eppendorf tubes, at 20 min intervals over up to 4 hours. Samples were immediately frozen in liquid nitrogen and stored in deep freeze until quantification.

Analyses of oxidized arachidonic acid derivatives were performed using sensitive gas chromatography-mass spectrometry in the negative ion chemical ionisation mode (NICI-GC-MS). Our data provide evidence for the presence of HETEs, 8-iso-$PGF_{2\alpha}$, $PGF_{2\_}$ and PGE_2 in microdialysate samples of normal skin, for their increase following UVB-irradiation and for the influence of diclofenac. Diclofenac was able to reduce the release of HETEs and 8-iso-$PGF_{2\_}$ to a different extent. Further investigations are necessary to show whether these new findings may also be relevant for therapeutical strategies in different inflammatory skin diseases.

Lipid oxidation products with high cardiovascular risk potential in chronic renal failure

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Reactive oxygen species are known to play an important role in the pathogenesis and progression of chronic renal failure. The aim of the investigations was to determine lipid oxidation products with high cardiovascular risk potential in serum samples of hemodialysis (HD) patients: F_2-isoprostanes (8-iso-$PGF_{2\alpha}$ and 9α,11α-$PGF_{2\alpha}$) as oxidized arachidonic acid isomers, major aldehydic peroxidation products 4-hydroxynonenal (HNE) and malonic dialdehyde (MDA) and various oxysterols. Serum samples of 110 hemodialysis patients and 80 healthy control persons were investigated. Isoprostanes were measured by GC-MS and HNE by TLC- and HPLC-separation of HNE-DNPH derivatives. MDA was assayed by HPLC of TBA-MDA adducts and oxysterols by GC of methylated TMS derivatives. Levels of all lipid oxidation products were markedly increased in patients in comparison to healthy control persons. Among oxysterols most pronounced increases were seen for α-triol, diene, ß-cholesterol epoxide and 7-keto compounds. The levels of 8-iso-$PGF_{2\alpha}$ and 9α,11α-$PGF_{2\alpha}$ increased by 50 and 100 % of control values. MDA and HNE levels correlate very well to the degree of renal anemia, whereas F_2-isoprostanes rather correlate to the concentration of the C reactive protein as one of the most important markers for inflammatory reactions.

Beta-carotene cleavage products induce oxidative stress by impairing mitochondrial functions: brain mitochondria are more sensitive than liver mitochondria

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In contrast to the well known beneficial antioxidative effects of carotenoids in some clinical studies, also harmful effects of carotenoid supplementation have been observed, e.g. a higher oxidative stress. The causal mechanisms are still unclear. Carotenoid cleavage products (CCPs) including highly reactive aldehydes and epoxides, which are formed during oxidative attacks in the course of antioxidative action, may be accused. Recently we published that beta-carotene cleavage products induce oxidative stress in vitro by impairing liver mitochondrial respiration (Siems et al. 2002. FASEB J. 16, 1289-1291). Here we extend these investigations to brain mitochondria. CCPs strongly inhibit the state 3 respiration of isolated rat brain mitochondria, even at concentrations between 0.5 and 20 µM. The inhibition of respiration was accompanied by a reduction of protein sulfhydryl content, decreasing glutathione levels and redox state, and elevated accumulation of malonic dialdehyde. All impairments by CCPs were much stronger in brain mitochondria than in liver mitochondria. The findings may reflect a basic mechanism of increasing the risk of cancer and other organ damages induced by carotenoid cleavage products, which are accumulated under conditions of heavy oxidative stress.

Status of antioxidant defense system during aging in rats liver

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The aim of this study is to determine what extent aging affects the status of antioxidant system during aging in rats and to evaluate the impact of supplementation with palmvitee (70% tocopherol, 30% tocotrienol) on the changes observed. The studies were conducted on young (3 and 9 month old) and aging (15 and 21 month old) male Wistar rats and involved procedures measuring liver tissue antioxidant enzymes, CuZn superoxide dismutase (SOD), and glutathione peroxidase (GPx). Measurements were also made on rats maintained on palmvitee supplemented diets. The data show that aging results in oxidative changes in liver tissue: a decline of tissue antioxidant enzyme, SOD was found in aging rats: 2.9 $\pm$ 0.07 and 2.8 $\pm$ 0.10 Units/mg protein respectively when compared to younger rats: SOD of 3.3 $\pm$ 0.16 and 3.4 $\pm$ 0.12 Units/mg protein respectively. However aging had a different effect on tissue antioxidant enzyme, GPx. An increase of GPx was observed with values of 9.9 $\pm$ 0.5 and 6.2 $\pm$ 0.55 specific activity/mg protein for older rats when compared to younger rats:3.2 $\pm$ 0.19 and 7.1 $\pm$ 0.76 specific activity/mg protein respectively. Supplementation with palmvitee however did not improve the activity of SOD with aging, but it however decreased the activity of GPx in young and aging rats when compared to non supplemented rats of the same age group. In this study, palmvitee may exert its antioxidant property by compensatory changes or "counter-balancing tendencies" for the antioxidant enzyme GPx but not for SOD in liver tissues.

Antioxidant activity of chickpea protein hydrolysates

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Lipid peroxidation is involved in many inflammatory and degenerative diseases, and is frequently responsible for deterioration of foods during storage and processing. Synthetic antioxidants such as butylated hydroxytoluene (BHT) are used as additives to prevent lipid peroxidation in foodstuffs. Although these antioxidants are very effective, there is concern about their safety. As part of an effort to develop inexpensive and safer antioxidants from natural sources, the antioxidant activity of peptides derived from food proteins is being studied. The specific goal of this study was to determine the antioxidant activity of chickpea protein hydrolysates obtained using alcalase, a food grade endoprotease preparation, and to characterize the peptide fractions responsible for this antioxidant activity. In time course experiments the highest antioxidant activity, determined using a β-carotene/linoleate oxidation model system, was found after 30 min of treatment with alcalase, which corresponds to a 30 % degree of hydrolysis. Two fractions with potent antioxidant activity were purified in a process involving ion-exchange, gel filtration, and reverse-phase chromatographies. These results show that hydrolysis of chickpea protein isolates with alcalase yields peptides with potent antioxidant activity.

Oral administration of Crataegus extraction protects against ischemia/reperfusion brain damage in the Mongolian gerbils

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Stroke is a serious neural disease. One of the important pathogeny is tremendous free radicals production during the ischemia/reperfusion period. In this work, Crataegus extraction (CE), a natural antioxidant extracted from the leave of *Crataegus pinnalifida Bge*,(containing both flavonoids (62.4%) and proanthocyanidines (27.2%)), was added to the drinking water of Mongolian gerbils for fifteen days before the animal suffered 5 minutes brain ischemic insult, to investigate the protective effect of CE on the ischemia/reperfusion brain damage. The reactive oxygen species (ROS), thiobarituric acid reactive substances (TBARS), the homogenate associated antioxidant level, the production of nitric oxide and nitrite/nitrate in the brain homogenate, were detected by ESR and spectrometer method. The protective effect of CE on the hippocampus CA1 area was determined by nissl staining, TUNEL, and transmission electron microscope (TEM). Results showed that pretreatment with the CE decreased ROS production, TBARS content, nitrite/nitrate concentration increased by IR damage, elevated the brain homogenate associated antioxidant level in a dose dependent manner. And pretreatment with CE increased the amount of available NO by elimination of the superoxide radical produced during reperfusion. There were more survival cells and less DNA damage in the hippocampal CA1 region of CE treated animals brain, testified by nissl staining, TUNEL, TEM method. These results suggest that oral administration of CE protects the brain against ischemia/reperfusion damage.

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