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OPENING LECTURE

OL-01
New insights into the biological significance of nitric oxide: cytochrome c interactions

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Nitric oxide (NO) reduces cytochrome c oxidase (CcO), the terminal enzyme of the mitochondrial electron transport chain, at concentrations below those at which it inhibits cellular respiration (VO_2). We have found that the CcO can be reduced by NO by up to $\sim$20% without affecting cell respiration. Partial reduction of the CcO by NO leads to accumulation of the reduced form of cytochrome c and to a consequent increase in the flux of electrons through the uninhibited population of the NO-free enzyme. In this way, cell respiration is maintained without compromising the bioenergetic state of the cell. We have demonstrated that reduction of the cytochrome c oxidase at relatively high [O_2] leads to the generation of reactive oxygen species (ROS) and to cell signalling, suggesting that ROS generation is not a consequence of hypoxia but of changes in the relative concentrations of NO and O_2. A further increase in the NO:O_2 ratio results in inhibition of cell respiration and a redistribution of O_2 both within and outside the cell, as well as to activation of glycolysis. These adaptations of cellular bioenergetics and metabolism to variations in the NO:O_2 ratio are likely to play an important role in the response of cells to stress leading to pathology.

SFRR-EUROPE LECTURE

SEL-01
Mitochondrial energy metabolism and redox signalling

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Mitochondrion-generated H_2O_2 is a signalling molecule that reports a high-energy charge to the cytosol and is involved in the redox regulation of mitogen activated protein kinase (MAPK) signalling pathways at different levels: activation of c-Jun N-terminal kinase (JNK) may be accomplished upon H_2O_2-stimulated dissociation of glutathione transferase-JNK complexes and/or inactivation of MAPK phosphatases. Upon activation (phosphorylation), JNK translocates to mitochondria and through a phosphorylation cascade regulates mitochondrial energy metabolism and apoptotic functions. This coordinated response encompasses mitochondrial retrograde signalling. Changes in mitochondrial metabolism upon translocation of JNK1 to mitochondria are effected by protein post-translational modification, namely phosphorylation. In addition to mitochondrial outer membrane protein targets, such as Bcl-2 and Bcl-xL, JNK (associated to the outer mitochondrial membrane) results in the phosphorylation (and inhibition) of the E1a sub-unit of the mitochondrial matrix pyruvate dehydrogenase complex, probably accomplished through an enhanced pyruvate dehydrogenase kinase 2 activity. In primary cortical neurons, this process results in a decrease in mitochondrion-generated ATP and compensatory increase in anaerobic metabolism, i.e. lactic acid. Pyruvate dehydrogenase activity is critical to the brain mitochondrial energy-conservation processes, for it links cytosolic glycolysis with the tricarboxylic acid cycle. Attempts to overcome an impaired pyruvate dehydrogenase activity (an early event in neurodegenerative disorders) involve alternative acetyl-CoA-generating pathways, such as ketone body oxidation; however, succinyl-CoA transferase—a requirement for ketone body oxidation in extrahepatic tissues—is sensitive to peroxynitrite and inhibited upon nitration; moreover, both pyruvate dehydrogenase- and succinyl-CoA transferase activities decline with ageing, thus limiting the generation of reducing equivalents channelled to oxidative phosphorylation. This energy–redox axis appears as a major regulatory device of mitochondrial and cell function.

CPL: CATHERINE PASQUIER LECTURE

CPL-01
Fingerprints of oxidative stress in age-related diseases: the biomarker–clinical marker association

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Oxidative stress is recognized as an important step in the pathophysiology of the major age-related diseases, including dementia and cardiovascular disease. Unfortunately, and paradoxically, too often advanced age ($>$75 years, i.e. the portion of the population mostly increasing in number currently) and presence of comorbidities constitute exclusion criteria from human studies on age-related diseases. Furthermore, there is still a huge gap between well-conducted clinical geriatric studies and high-level basic research on oxidative stress. Finally, studies aimed at characterizing oxidative stress in the major diseases of ageing usually focus on single symptoms, endpoints or outcome measures and often disregard a broad evaluation of oxidative stress biomarkers/antioxidant micronutrients and a comprehensive geriatric, including nutritional, assessment. As a consequence, there are still a paucity of clear-cut data on the causative role of oxidative stress in disease development as well as frustration for the results of nutritional and antioxidant interventions against cardiovascular disease and dementia, among others. In our experience, not only specific micronutrient profiles are identified according to a given disease status, but there are also specific associations between biomarkers of oxidative stress/antioxidant micronutrients and clinical reliable, non-invasive markers of disease. Biomarkers of oxidative stress are indeed required to improve our diagnostic sensitivity and specificity and to monitor the biological activity of cardiovascular and neurodegenerative diseases in terms of the burden of cellular involvement and the tempo of disease progression. The link between biological markers of oxidative stress and clinical indicators of disease severity and outcome might help in identifying populations at risk and further refining levels of micronutrients required for optimum long-term health.

CLOSING LECTURE

CL-01
Free radicals in skeletal muscle: Essential signals or mediators of damage

Malcolm J. Jackson, Deborah Pye, Jésus Palomero, Tabitha Kabayo, Graeme Close, Aphrodite Vasilaki, & Anne McArdle
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Studies over the last 25 years have examined the generation of free radicals and other reactive oxygen species (ROS) in skeletal muscle and the manner in which this is altered during both physiological (exercise) and pathological processes. Initial investigations concentrated almost entirely on the potential deleterious effects of ROS and appeared at odds with the acknowledged beneficial effects of regular exercise on health. It is now clear that during contractile activity skeletal muscle cells generate increased amounts of reactive oxygen species (ROS) through a number of different pathways and that these ROS modulate at least some of the adaptive responses that occur in skeletal muscle following contractile activity. This process involves activation of redox-regulated transcription factors, such as AP-1, NFκB and HSF-1 and leads to increased expression of cytoprotective proteins that protect muscle cells against potential damage following subsequent rises in ROS activity. In contrast to these apparently positive roles of ROS, there is also evidence that, during ageing, this ability of skeletal muscle to adapt to contraction-induced ROS fails with consequent increase in oxidative damage that is associated with the age-related loss of muscle mass and function.

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SESSION I—CELLULAR THIOL REDOX HOMEOSTASIS

S1-01
Redox regulation of inflammation and metabolic stress by TRX/TBP-2 system

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Thioredoxin-1/TRX1 is a small ubiquitous protein with redox active cysteins and plays an important role in regulating cell proliferation, apoptosis and the activation of transcription factors by reducing intracellular molecules. During the search for the target molecules interacting with TRX1, we have identified thioredoxin binding protein-2 (TBP-2), also referred as VDUP1 or Txnip, in a yeast two hybrid assay (1). TBP-2 is induced by vitamin D3, glucose, lipopolysaccharide (LPS) and ligands of peroxisome proliferators-activated receptor-g and likely to be involved in multiple cellular processes such as redox regulation, cancer suppression, NK cell development and metabolic control. TBP-2 knockout (TBP-$2^{-/-}$) mice are predisposed to death with bleeding tendency, hypertriglycemia, hyperinsulinemia and hypoglycemia as a result of fasting as well as intraperitoneal administration of LPS (2). The fasting- or LPS-induced death was rescued by supplementation of glucose but not by that of oleic acid, indicating that inability of fatty acid utilization plays an important role in the anomaly of TBP-$2^{-/-}$ mice. Furthermore, we have also established TBP-2 over-expressing mice, which showed a variety of phenotypes involving immune deviation, enhanced oxidative stress and lipid mobilization. These findings provide clear evidence that TBP-2 has an important role in the regulation of energy-utilizing pathways and the cellular redox status as well as innate immune response. Recently we found that macrophage migration inhibitory factor (MIF) binds to TRX1 in extra- and intra-cellular compartments (3), suggesting regulation of multiple cellular processes by a cross-talk between TRX1, TBP-2 and MIF molecules.

(1) Identification of thioredoxin-binding protein-2/vitamin D3 up-regulated protein 1 as a negative regulator of thioredoxin function and expression [1];
(2) Impaired fatty acid utilization in thioredoxin binding protein-2 (TBP-2)-deficient mice: a unique animal model of Reye syndrome [2]; and
(3) Human thioredoxin-1 ameliorates experimental murine colitis in association with suppressed macrophage inhibitory factor production [3].

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S1-02
Thiols in immunity: From oxidative stress to redox regulation

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Within the concept of oxidative stress, glutathione (GSH) acts as an antioxidant to scavenge reactive species and its depletion enhances oxidative damage. This has often been associated with a view of GSH as an anti-inflammatory agent as thiol antioxidants inhibit NF-kB and production and action of cytokines including TNF and IL-1. However, in the context of redox regulation, GSH is a signalling molecule and its depletion is detrimental because it blocks a regulatory mechanism. One means of redox regulation by GSH and other thiol antioxidants is through protein glutathionylation. We used redox proteomics to identify proteins undergoing oxidative stress in primary T lymphocytes and identified some key proteins such as cyclophilin A, the ligand of the immunosuppressor cyclosporin. To demonstrate that GSH is used in signalling, we exposed HL60 monocytic cells to H_2O_2 with and without GSH-depleting agent buthionine sulphoximine and analysed gene expression using microarrays. The analysis revealed 2016 genes regulated by H_2O_2. Of these, 215 genes showed GSH-dependent expression changes, classifiable into four clusters displaying down-regulation or up-regulation by H_2O_2, either augmented or inhibited by GSH depletion. These clusters of GSH-regulated genes included several cytokine and chemokine ligands and receptors. Thus, GSH, in addition to its antioxidant and protective function against oxidative stress, has a specific signalling role in redox regulation.

S1-03
Thioredoxin and glutaredoxin systems in cellular thiol redox homeostasis

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Reactive oxygen and nitrogen species are small molecules which are implicated in cell signalling. It was shown that the protein disulphide reductase activity of the thioredoxin (Trx) system, containing Trx, thioredoxin reductase (TrxR) and NADPH can be inhibited by incubation with S-nitrosoglutathione (GSNO). We hypothesized this effect could result from S-nitrosylation of human Trx1 which, apart from the Cys 32 and Cys 35 in the active site, have three structural Cys residues in position 62, 69 and 73. The number of nitrosothiols in fully reduced Trx1 treated with GSNO was calculated from the spectra of reduced and nitrosylated samples, using a molar extinction coefficient of $920\,M^{-1}\,cm^{-1}$ at 335 nm, showing 2.08 ± 0.19 nitrosothiols. Different Cys to Ser mutants and wild-type Trx1 were nitrosylated to find the localization of nitrosothiols. We show that Cys 69 and Cys 73 became nitrosylated and Cys 32 and Cys 35 in the active site were oxidized by the formation of a disulphide bridge. The protein disulphide reductase activity of Trx was inhibited after nitrosylation of two Cys 69 and Cys 73; however, this effect was reversible and Trx regained its activity after a lag phase. H_2O_2 also inhibited Trx activity which was regained after a lag phase; however, a different mechanism was involved that is the formation of a second disulphide between Cys 62 and Cys 69. Interestingly, Cys 73 was not affected by H_2O_2 and no dimerization or oligomerization was observed. Mammalian cytosolic TrxR was not affected by either GSNO or H_2O_2. The transient inhibition of human Trx1 activity by H_2O_2 and NO probably plays a role in cell signalling via providing time for the transmission of oxidative and/or nitrosative signals like reversible inhibition of phosphotyrosine phosphatases. Glutaredoxin (Grx) catalyses GSH-dependent oxidoreductions and is also subject to extensive oxidation and S-nitrosylation reactions with striking differences between the cytosolic/nuclear Grx1 and the mitochondrial Grx2a [1].

Reference

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S1-04
Changes in nuclear glutathione during the cell cycle

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Nuclear glutathione plays a major role in cell physiology. We have studied the changes in nuclear glutathione distribution and the progression of cell cycle. The former was studied by confocal microscopy using CMFDA (5-chloromethyl fluorescein diacetate) and the latter by flow cytometry and protein expression of cell cycle regulatory proteins. In proliferating cells, when 41% of them were in the S + G2/M phase of the cell cycle, GSH was located mainly in the nucleus. When cells reached confluence (G_0/G_1) it was localized in the cytoplasm, with a perinuclear distribution. The nucleus/cytoplasm fluorescence ratio for GSH reached a maximal mean value of 4.2 ± 0.8 6 hours after cell plating. A ratio higher than 2 was maintained during exponential cell growth. In the G_0/G_1 phase of the cell cycle the nucleus/cytoplasm GSH ratio decreased to values close to one. Nuclear glutathionilated proteins increased in the early phases of cell proliferation, when higher GSH levels were present in the nucleus. Oxidized nuclear proteins reached higher levels when cells where confluent, i.e. when nuclear GSH levels decreased. We report here that cells concentrate GSH in the nucleus, in early phases of cell growth, when most of the cells are in an active division phase, and that it redistributes uniformly between nucleus and cytoplasm when cells reach confluence.

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SESSION II—PROTEIN POST-TRANSLATIONAL MODIFICATIONS IN DISEASE

S2-01

Protein tyrosine nitration in hydrophilic and hydrophobic biocompartments: Mechanisms and relevance on cell death

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Protein tyrosine nitration is a post-translational modification found *in vivo*, secondary to excess formation and reactions of nitric oxide-derived oxidants. In addition to its possible effects on protein structure and function, tyrosine nitration is being revealed as a biomarker and even a risk factor in a variety of disease conditions including cardiovascular, inflammatory and neurodegenerative disorders. Formation of protein 3-nitrotyrosine depends on free radical mechanisms and, importantly, nitration yields are responsive to increases of either superoxide/hydrogen peroxide or nitric oxide levels; however, nitration yields *in vivo* are typically low, mainly due to the presence of strong reducing systems (e.g. glutathione), that can potently inhibit at different levels the nitration process. Notably, tyrosine nitration in some proteins (e.g. cytochrome c, nerve growth factor, HSP90) can induce a toxic 'gain-of-function' that can trigger pro-apoptotic processes. Recent studies using hydrophobic tyrosine analogues and tyrosine-containing peptides reveal that factors controlling nitration in hydrophobic environments such as biomembranes and lipoproteins can differ to those in aqueous compartments. In particular, exclusion of key soluble reductants from the lipid phase will more easily permit nitration while lipid-derived radicals are suggested as important mediators of the one-electron oxidation of tyrosine to tyrosyl radical in proteins associated to hydrophobic structures. Development and testing of hydrophilic and hydrophobic tyrosine probes that can compete with endogenous constituents for the nitrating intermediates provide tools to define nitration mechanisms *in vitro* and *in vivo* and can also serve cell and tissue protective functions against the toxic effects of protein tyrosine nitration.

S2-02

Regulation of plasma membrane permeability to H_2O_2 during adaptation to H_2O_2

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H_2O_2 diffuses across biomembranes much faster than other reactive oxygen species, which lead to the view that H_2O_2 diffusion across biomembranes is not rate-limiting (i.e. is 'free'). Here we present evidence that H_2O_2 does not freely diffuse across biomembranes and, more importantly, H_2O_2 regulates biomembrane properties and composition. (1) H_2O_2 diffusion is relatively slow; e.g. it is about one order of magnitude slower than water. (2) Nevertheless, H_2O_2 diffusion across biomembranes is fast enough to reach steady-state dynamical equilibration between different cellular compartments in the order of seconds. (3) For most cell types, H_2O_2 diffusion into the cell is slower than the rate of H_2O_2 removal by antioxidant enzymes. That is, H_2O_2 diffusion is partially rate-limiting and, therefore, gradients are formed when the source of H_2O_2 is separated from the sink by a biomembrane. (4) In *Saccharomyces cerevisiae* (SC) there is a correlation between this gradient across the cellular membrane and resistance to H_2O_2. (5) In SC the plasma membrane is not a passive actor in the defense against H_2O_2 but its permeability is down-regulated by H_2O_2 and other agents. (6) In SC, during adaptation to H_2O_2, plasma membrane undergoes several changes: fluidity decreases, squalene and very-long fatty acids increase and phosphatidylethanolamine decreases while phosphatidylcholine increases. (7) Down-regulation of fatty acid synthase increases the resistance of SC to H_2O_2. In conclusion, non-lethal H_2O_2 doses control the properties and composition of plasma membrane by a multi-factorial process involving several pathways.

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S2-03

Proteolytic degradation of oxidized and AGE (advanced glycation endproduct)-modified proteins

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Mammalian cells contain a multitude of proteolytic enzymes characterized by different compartmentalization and substrate specifity. One of the major proteolytic systems is the proteasomal system. Oxidatively modified proteins are selectively recognized and degraded by the proteasomal system. The isolated 20S proteasome is able to degrade moderately oxidized proteins, whereas severe oxidized model proteins are poor substrates of this protease. A multitude of globular proteins show this behaviour after oxidation. The recognition of oxidized proteins by the 20S proteasome is due to unfolding. Unfolded proteins seem to be in general degraded by the 20S proteasome. In living mammalian cells it was demonstrated that the proteasome is the proteolytic system responsible for the degradation of oxidized proteins and that the exposure of cells to oxidants is followed by an enhanced protein turnover. The role of the ubiquitin system in the recognition of oxidized proteins was tested. No clear evidence for the ubiquitination of oxidized proteins could be found so far. Another post-translational modification of many proteins are AGE-modifications. Interestingly, in several diseases AGE-modified proteins accumulate, perhaps due to a bad proteolytic susceptibility of AGE-modified proteins. On the other hand we could clearly demonstrate that carbohydrate modification does not necessarily lead to a decline in proteasomal/protease degradation. It is increasingly recognized that malfunction of the proteasomal system is playing a major role in several diseases (including neurodegenerative diseases) and the ageing process.

S2-04

Oxidation of reactive thiols in disease

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Vascular disease is accompanied by increased formation of oxidants that can inhibit protein function or target the protein for degradation. However, lower levels of reactive oxygen and nitrogen species also serve as signalling molecules in normal cells by altering the electrical charge or structure of proteins. The cysteine thiol can account for both responses to oxidants as their modifications range from reversible to irreversible. The cysteine residues most affected are those that exist as thiolate anions at physiological pH, and are therefore more reactive. As a prime example of this bimodal thiol function, the sarco(endo)plasmic Ca^{2+}ATPase (SERCA) is *S*-glutathiolated by peroxynitrite, which is formed from nitric oxide released from endothelial cells when it reacts with superoxide anion in smooth muscle cells. The 307 Da, positively-charged glutathione adduct increases SERCA Ca^{2+} uptake activity and decreases intracellular Ca^{2+} levels. When the most reactive SERCA cysteine, C674, is mutated to serine the normal activation by nitric oxide is prevented. In diseased arteries SERCA-C674 is irreversibly oxidized and, like the mutant, fails to respond to nitric oxide. Thus, low concentrations of oxidants produce specific protein modifications that serve a signalling function, but exposure of cells to higher concentrations for longer periods cause irreversible changes and protein dysfunction. Treatment of oxidant stress will require further understanding of the normal and pathological functions of reactive oxygen and nitrogen species.

SESSION III—INTERORGANELLE CROSS-TALK CONTROL OF APOPTOSIS

S3-01

Conformational alterations regulate the subcellular localization of cytochrome *c*

Luiz Godoy[1], Cristina Muñoz Pinedo[2,3], Laura Castro[4], Verónica Tórtora[4], Christopher Schonhoff[1], Michael King[1], Simone Cardacci[1,5], Qian Miao[1], Jian Fei Jiang[6], Alexandr Kapralov[6], Ron Jemmerson[7], Gary G. Silkstone[8], Jinal N. Patel[9], James Evans[9], Michael T. Wilson[8], Douglas R. Green[2], Valerian E. Kagan[6], Rafael Radi[4], & Joan B. Mannick[1]

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In its native conformation, cytochrome *c* functions in electron transport in mitochondria and in caspase activation in the cytoplasm. However, the possibility that conformational alterations confer additional functions to cytochrome *c* has not been explored. In its native state, cytochrome *c* has a compact tertiary structure with a hexacoordinated heme iron. However, the methionine 80 (M80)-Fe ligation of cytochrome *c* is weakened or ruptured when M80 is oxidized [1,2], when cytochrome *c* is tyrosine nitrated [3,4] and/or when cytochrome *c* interacts with anionic phospholipids such as cardiolipin, a phospholipid in the inner mitochondrial membrane [5–7]. To model this conformational alteration and determine if disruption of the M80-Fe ligation confers new functions to cytochrome *c*, a cytochrome *c* mutant was expressed in cells in which M80 is mutated to an alanine (M80A), resulting in constitutive disruption of the M80-Fe ligation. Here we show that M80A cytochrome *c* has increased peroxidase activity and is spontaneously released from mitochondria and translocates to the cytoplasm and nucleus in the absence of apoptosis via a Bax/Bak and mitochondrial permeability transition pore (MPTP)-independent mechanism. Moreover, M80A may be modelling the behaviour of endogenously oxidized or nitrated cytochrome *c* because treatment of cells peroxynitrite, an endogenously produced oxidant associated with both M80 oxidation and tyrosine nitration of cytochrome *c* [3,4], induces the translocation of WT cytochrome *c* to the cytoplasm and nucleus in the absence of apoptosis. Further, cells expressing M80A have increased rates of proliferation in response to low levels of nitrative stress. Our findings raise the possibility that endogenous conformational alterations, such as tyrosine nitration or M80 oxidation, that disrupt the M80-Fe ligation of cytochrome *c* stimulate the translocation of cytochrome *c* from mitochondria to the cytoplasm and nucleus, where cytochrome *c* may regulate the response of cells to nitrative stress. Thus, cytochrome *c* may have previously unrecognized functions that are dependent on its conformation as well as its sub-cellular localization.

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S3-02

Neuroprotection by PARP-1 during endogenous oxidative stress

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In neurons, DNA is prone to free radical damage, although repair mechanisms preserve their genomic integrity. However, activation of the DNA repair system, poly(ADP-ribose) polymerase (PARP-1), is thought to cause neuronal death through NAD^+ depletion and mitochondrial membrane potential ($\Delta\psi_m$) depolarization. Here, we show that abolishing PARP-1 activity in primary cortical neurons can either enhance or prevent apoptotic death, depending on the intensity of an oxidative stress. Only in severe oxidative stress PARP-1 activation results in NAD^+ and ATP depletion and neuronal death. To investigate the role of PARP-1 in an endogenous model of oxidative stress, we used an RNA interference (RNAi) strategy to specifically knock down glutamate-cysteine ligase (GCL), the rate-limiting enzyme of glutathione biosynthesis. GCL RNAi spontaneously elicited a mild type of oxidative stress that was enough to stimulate PARP-1 in a Ca^{2+}-calmodulin kinase II-dependent manner. GCL RNAi-mediated PARP-1 activation facilitated DNA repair, although neurons underwent $\Delta\psi_m$ loss followed by moderate apoptotic death. PARP-1 inhibition did not prevent $\Delta\psi_m$ loss, but enhanced the vulnerability of neurons to apoptosis upon GCL silencing. Conversely, mild expression of PARP-1 partially prevented from GCL RNAi-dependent apoptosis. Thus, in mild progressive damage likely occurring in neurodegenerative diseases, PARP-1 activation plays a neuroprotective role that should be taken into account when considering therapeutic strategies.

S3-03

Dual role of NADPH oxidases in the regulation of apoptosis by TGF-beta in hepatocytes

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TGF-beta induces apoptosis in hepatocytes through a mechanism dependent on the production of reactive oxygen species (ROS), which mediate loss of mitochondrial-transmembrane potential, release of cytochrome c and caspase activation [1]. Looking for the origin of ROS, we found that TGF-beta activates a NADPH oxidase-like system by a mechanism dependent on protein synthesis [2]. However, TGF-beta also induces survival signals in foetal rat hepatocytes and hepatoma cells, through transactivation of the epidermal growth factor receptor (EGFR) [3,4]. A detailed analysis of the molecular mechanisms that mediate up-regulation of the EGFR ligands by TGF-beta in hepatocytes have revealed the potential involvement of ROS [5]. TGF-beta mediates activation of the NF-κB pathway, which is inhibited by DPI (NADPH oxidase inhibitor) and is required for TGF-beta-induced up-regulation of TGF-alpha and HB-EGF. Different members of the NADPH oxidase family of genes are expressed in hepatocytes, included *nox1*, *nox2* and *nox4* [6]. TGF-beta induces both *nox4* and *nox1* and activates Rac1. Recent experiments with specific siRNA, to knock-down Nox1 or Nox4, have indicated that Nox4 is required for TGF-beta induced cell death, whereas Nox1 might play a survival role. Considering all these results together, ROS produced by NADPH oxidases might mediate both pro- and anti-apoptotic signals in TGF-beta-treated cells. Possible implications in hepatocarcinogesis will be discussed.

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SESSION IV—NEURODEGENERATION AND AGEING

S4-01

Heme oxygenase-1: Controlling the pro-oxidant effects of free heme during neuroinflammation

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Inflammation is a beneficial response to injury and/or infection that is essential to the initiation of immune responses. Unfettered inflammation, however, can lead to irreversible tissue injury and to the development of so-called 'inflammatory diseases', including autoimmune neuroinflammation, e.g. multiple sclerosis and lethality arising from malaria infection, e.g. cerebral malaria. Under inflammatory conditions, the ubiquitous stress-responsive enzyme heme oxygenase-1 (HO-1 encoded by the gene *Hmox1*) acts as the rate-limiting enzyme in the catabolism of heme, yielding equimolar amounts of free Fe, carbon monoxide (CO) and biliverdin. Free Fe induces the expression of the Fe sequestering protein heavy chain ferritin (H-ferritin). As presented in this talk the pathogenesis of both neuroinflammatory diseases is very significantly exacerbated in *Hmox1* deficient vs wild type mice. This reveals that the pathogenesis of these apparently disparate pathologic conditions actually shares common mechanisms, namely inhibition of their pathologic outcome by HO-1. Another common feature shared by these diseases seems to be the release of heme from hemeproteins, a process that leads to the generation of deleterious free heme. It is well established that the Fe molecule contained within free heme can act as a potent Fenton reactor, which, as shown, hereby becomes highly pro-apoptotic when combined with several pro-inflammatory agonists. The pro-apoptotic effect of free heme is strictly linked to its pro-oxidant activity. HO-1 counters these deleterious effects through at least three mechanisms, including: (i) heme degradation exerted by HO-1 itself, (ii) inhibition of heme release from hemoproteins exerted by CO and (iii) Fe sequestration by H-ferritin. Presumably all three functions counter the pathologic outcome of autoimmune neuroinflammation as well as cerebral malaria. The implications of these findings will be discussed, taking into consideration their potential therapeutic application as well as the existence of a broadly expressed polymorphism in the regulatory region of the human HMOX1 gene, dictating susceptibility to inflammatory diseases as well as response to therapy.

S4-02

Redox regulation of celular stress response in ageing and neurodegenerative disorders: Role of vitagenes and modulation by antioxidants

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Oxidative damage plays a crucial role in the brain ageing process [1]. Induction of heat shock proteins (HSPs) is critically utilized by brain cells in the repair process following various pathogenic insults [1,2]. When appropriately activated, the heat shock response has the capability to restore cellular homeostasis and rebalance redox equilibrium. Activation of antioxidant pathways is particularly important for neural cells with relatively weak endogenous antioxidant defences [2]. We have recently focused our research on the role of acetylcarnitine (LAC) in the defense mechanisms against cellular stress and neurodegeneration [3]. In the present study we investigated mRNA expression and protein synthesis of Hsps and the oxidant status in adult (12 months) and senescent (28 months) rats and the effect of LAC treatment in senescent rats. mRNA and protein synthesis of Hsps increased in senescent rats compared to adults in all brain regions examined; the maximum increase was observed in the hippocampus followed by cerebellum, cortex and striatum. Hsps increase was associated with significant decrease in glutathione (GSH) redox state and decrease of carbonyls and HNE content. Interestingly, treatment with LAC resulted in a marked increase of heme oxygenase-1 (HO-1), Hsp70 and mtSOD expression, of GSH content and a decrease of HNE and protein carbonyl contents in the hippocampus, striatum, cortex and cerebellum. These results were also confirmed by *in situ* hybridization experiments. We used also a parallel redox proteomic approach [4–8] to identify the proteins that are oxidized in aged rat brain and those proteins that are protected by LAC treatment. Redox proteomics analysis in HP and CX, brain regions in which all indices of oxidative modification are elevated in brain ageing showed that the specific carbonyl levels of three proteins, hemoglobin (HMG), cofilin 1 (COF 1) and beta-actin (ACT), are significantly increased in HP of senescent rats. Carbonyl levels of these proteins are reduced by LAC administration in old rats brains. In the CX of senescent rats, the specific carbonyl levels of seven proteins were increased. These proteins are heat shock protein 70 (Hsp70), glyoxylase 1 (GOL 1), beta-actin (ACT), 3-mercaptopyruvate sulphurtransferase (MPST), peroxiredoxin 1 (PDX), phosphoribosyl pyrophosphate synthetase 1 (PPRPS1) and fumarase (FUM). LAC administration reduced the specific carbonyl levels of all these protein in the CX of senescent rats. The proteins identified in our study are involved in three processes which are impaired in aged brains: antioxidant defence, mitochondrial function and plasticity. LAC treatment might improve the decline of these functions. We posit that LAC should be considered as a potential therapeutic contributor for the treatment of cognitive decline in ageing and age-related neurodegenerative disorders. In conclusion our results sustain a role for a redox-dependent modulation of Hsp expression occurring in the ageing brain. Notably, increased Heat shock protein expression, by promoting the functional recovery of oxidatively damaged proteins, protects brain cells from progressive age-related cell damage [5,6]. Therefore, therapeutic strategies focusing on acetylcarnitine treatment, by up-regulating HO-1 and Hsp70 signal pathways may represent a crucial mechanism of defence against free radical-induced damage of specific proteins occurring in ageing brain and in neurodegenerative disorders [7,8]. These findings are relevant to potential pharmacological strategy pointing to maximize cellular stress resistance in vulnerable tissues such as the brain and thus providing neuroprotection [6–10].

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S4-03

Mitochondrial dysfunction in rat hippocampus upon ageing

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Hippocampal mitochondria of aged (12 months) and senescent (20 months) rats showed, when compared with young (4 months) rats, marked decreases in the rate of respiration in state 3 with NADH-dependent substrates (51%), and in the activities of mitochondrial complexes I (73%) and IV (54%). The activity of mtNOS also decreased with age, up to 66% at 20 months. The referred decreases in electron transfer, respiration and mtNOS activity were more marked in hippocampus than in brain cortex or whole brain. The histochemical assays of complexes I and IV in the hippocampus showed a detrimental effect of ageing. Oxidative damage, measured by TBARS and protein carbonyls, increased in aged and senescent hippocampus (66–74% in TBARS and 48–96% in carbonyls, respectively). The oxidative damage and the reduction of the mitochondrial capacity for energy production in rat hippocampus, more marked than in the cortex or whole brain, indicates that hippocampus is selectively affected in mammalian ageing. The ageing of rat hippocampus is associated with impaired electron transfer, mitochondrial dysfunction and oxidative damage.

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S4-04

Neurodegeneration in Alzheimer's disease: Inter-organelle cross-talk and oxidative stress in cell life and death decisions

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Recent molecular, cellular and gene expression studies have revealed that a link between Aβ peptides, the generation of free radicals and mitochondrial oxidative damage are key factors in Alzheimer's disease (AD) development and progression. An increasing body of evidence supports that endoplasmic reticulum (ER) stress mediates neurodegeneration and is relevant in AD pathogenesis. We have demonstrated, in cultered cortical neurons, that Aβ peptides cause ER stress. An early and sustained increase in ER-derived intracellular calcium was observed, as well as the generation of ROS and the activation of the mitochondrial apoptosis pathway. These effects were inhibited by blocking the major ER calcium release channels and the antioxidants CoQ10 and vitamin E where shown to prevent ROS generation and mitochondria dysfunction under ER stress conditions. In cytoplasmic hybrid (cybrid) cells, obtained by the fusion of mitochondria DNA (mitDNA)-depleted human teratocarcinoma cells (NT2 $\rho 0$ cells) with platelets from sporadic AD patients, we could clearly demonstrate that the mitochondrial dysfunction present in AD patients (inhibition of mitochondrial respiratory chain complex IV) renders the cells more vulnerable to Aβ-induced ER stress. More recently, in an AD mouse model, the triple transgenic mice ($3 \times$ Tg-AD), we observed that oxidative stress is an early event during the progression of the amyloid pathology. In this AD animal model, before Aβ deposition in brain amyloid plaques, a depletion of vitamin E, an increase in lipid peroxidation and an impairement of the glutathione cycle were shown to occur. Altogether our results demonstrate that a close connection between ER and mitochondria is crucial in neuronal death decision in AD, ROS and calcium being key players in the inter-organelle cross-talk.

S4-05

Does superoxide-induced mitochondrial dysfunction lead to muscle atrophy?

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Oxidative stress and mitochondrial ROS production have been proposed to play a significant role in muscle atrophy. Muscle mitochondrial ROS generation is elevated in old vs young mice, in mice lacking CuZnSOD (*Sod1*$^{-/-}$) and in the neurodegenerative disease ALS, conditions associated with increased oxidative stress and muscle atrophy. ROS generation is increased 40% in old wild type mice associated with a 30% loss in gastrocnemius mass. In *Sod1*$^{-/-}$ mice, ROS production is increased over 100% in 20 month old mice and muscle mass is decreased by 50%. ALS G93A mutant mice show a 75% loss of muscle mass during disease progression and up to 12-fold higher muscle mitochondrial ROS generation, Conversely, in a second ALS mutant model, H46RH48Q mice, ROS production is increased only $\sim$4-fold along with a less dramatic loss (30%) in muscle mass. Thus, ROS production is strongly correlated with the extent of muscle atrophy in these models. To determine if denervation plays a role in initiating ROS generation in muscle mitochondria, we measured ROS production in muscle 7 days following sciatic nerve transection. Mitochondrial ROS production increased nearly 30-fold with denervation. Changes in mitochondrial ROS generation are parallelled by increases in phospholipase A_2 (PLA_2) activity and inhibition of PLA_2 reduces ROS production. Caloric restriction in the *Sod1*$^{-/-}$ mice maintains muscle mass, reduces PLA_2 and reverses the increase in mitochondrial ROS production. We conclude that enhanced generation of mitochondrial ROS may be a common factor in the mechanism underlying denervation-induced atrophy and that pro-inflammatory pathways may mediate these effects.

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SESSION V—ANTIOXIDANTS AND REDOX SIGNALLING

S5-01

Transcriptional activation of redox sensitive genes in vascular endothelium

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To counteract oxidative stress cells have evolved defence mechanisms involving the induction of detoxifying enzymes and antioxidant stress proteins, regulated via interaction of the redox sensitive transcription factor Nrf2 with a *cis*-acting element designated as *antioxidant response element* (*ARE*)/*electrophile response element* (*EpRE*). Keap1 normally sequesters Nrf2 in the cytoplasm in association with the actin cytoskeleton, facilitating proteosomal degradation and, under conditions of oxidative stress, Nrf2 translocates to the nucleus leading to gene transcription. We previously reported abnormalities in Ca^{2+} signalling in human foetal endothelial (HUVEC) and smooth muscle (HUASMC) cells isolated from pre-eclamptic (PE) pregnancies [1,2]. Our recent studies in HUVEC have revealed that homocysteine, 4-HNE and angiotensin increase $O_2^{-\cdot}$ production in normal cells but fail to activate NADPH oxidase in PE cells. Impaired glucose-6-phosphate dehydrogenase activity in PE HUVEC results in decreased intracellular NADPH levels and impaired redox cycling [3]. Under basal conditions, expression of the antioxidant protein HO-1 is low in normal and PE HUVEC and, although HNE evokes a dose-dependent increase in HO-1 expression in normal HUVEC, induction is markedly attenuated in PE cells. Similarly, Nrf2 nuclear translocation and induction of HO-1 are significantly reduced in PE HUASMC. Our findings implicate Nrf2 in ROS mediated antioxidant gene expression in foetal vascular cells and suggest that Nfr2-mediated signalling *is impaired* in pre-eclampsia. As these phenotypic changes persist in culture, they may have implications for long-term 'programming' of the foetal cardiovascular system.

Supported by British Heart Foundation and BBSRC.

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S5-02

Cell signalling by lipid oxidation products

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Among the compounds generated via lipid oxidation reactions in low density lipoproteins (LDL) and subsequently found in the atherosclerotic plaque are oxysterols, core-aldehydes, 4-hydroxyalkenas, whose ability of signalling to the nucleus of cells has been intensively investigated in our laboratory over the last years. All three classes of compounds include candidate molecules for a role in the pathogenesis of vascular remodelling which takes place during atherosclerosis progression, because of their pro-fibrogenic, pro-inflammatory, pro-apoptotic effects. MAPK pathway appears involved in all signalling effects so far observed with these lipid oxidation products. With particular regard to vascular cells, MEK/ERK phosphorylation resulted to be selectively stimulated, at pathophysiologically relevant concentrations, by various oxysterols and by 9-oxononanoyl cholesterol (9-ONC), the most represented cholesterol core-aldehyde in LDL. On the contrary, 4-hydroxynonenal (HNE), a major end-product of not enzymatic oxidation of n-6 polyunsaturated fatty acids, selectively up-regulated JNK. In relation to gene transcription promoted by the investigated lipid oxidation products, defined oxysterols, including 7α-hydroxycholesterol, were shown to enhance nuclear translocation of NF-kB and to activate PPARγ, while 9-ONC and HNE markedly activated AP-1, but not NF-kB. The contribution of other LDL lipid oxidation products to the progression of atherosclerosis will be quoted and discussed.

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S5-03

The endothelial cell as a target for nitroxidative stress

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The capacity of nitric oxide (NO) to regulate cellular signalling relies in part on its ability to produce covalent protein post-translational modifications (PTMs) in conjunction with other reactive oxygen and nitrogen species, a phenomenon known as nitroxidative stress. NO can promote both the modification of thiols by incorporation of NO (S-nitrosylation) or glutathione (S-glutathionylation). Both modifications are produced by different reactions induced by nitric oxide-related species. Furthermore, the non enzymatic reaction of NO with the superoxide radical may yield peroxynitrite ($ONOO^-$), which, through the formation of other intermediate species, may promote tyrosine nitration, another PTM linked to the regulation of protein function. Using different approaches we have attempted to identify PTMs related to nitroxidative stress in endothelial cells, which are archetypal NO generators. Among these, we focused on S-nitrosylation of Hsp90 and nitration of MnSOD, and we have been able to assign functional consequences to each of these PTMs. We have also recently identified S-glutathionylation of GAPDH as a redox-sensitive mechanism which regulates endothelin-1 mRNA stability. *In vitro* experiments confirmed that GAPDH binds specifically to adenine and uridine-rich elements (AREs) of ET-1 mRNA. Selective targeting of endothelial cell GAPDH by siRNA upregulated ET-1 expression by 3′-UTR-mediated mRNA stabilization. Using recombinant heterologous GAPDH we found that catalytic Cys152 participates in the binding to AREs and that specific S-glutathionylation abrogated this capacity, an action that may occur also *in vivo* under oxidative stress conditions. Hence, this PTM may represent a novel mechanism coupling endothelial gene expression to its regulation by nitroxidative stress.

S5-04

Protective effect of hydrazine derivatives in atherosclerosis

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Reactive carbonyl compounds (RCC) generated by the oxidation of polyunsaturated fatty acids and by sugar glycoxidation, namely Advanced lipid peroxidation end products (ALEs) and Advanced Glycation end products (AGEs), respectively, accumulate with ageing and oxidative stress-related diseases, such as atherosclerosis, diabetes or neurodegenerative diseases. These compounds alter progressively the function of cellular and tissular proteins by forming adducts on free amino groups and thiol residues, thereby inducing the 'carbonyl stress'. Free radical scavengers and antioxidants prevent the generation of lipid peroxidation products, but are inefficient on pre-formed RCCs. Conversely, carbonyl scavengers prevent carbonyl stress by inhibiting the formation of protein cross-links, but exhibit poor antioxidant activities. We report here the carbonyl scavenger and anti-atherogenic properties of several hydrazine derivatives, including aminoguanidine and the anti-hypertensive drug hydralazine. Hydrazine derivatives inhibited oxidized LDL (oxLDL) and 4-HNE-mediated cytotoxicity and (at a lesser extent) the oxidation of LDL mediated by cultured vascular cells and the formation of foam cells. These drugs prevented the carbonyl stress induced by oxLDL and 4-hydroxynonenal (4-HNE) and inhibit the decrease in the free amino group content of cellular proteins, the increase in carbonylated proteins and the subsequent protein dysfunctions. As an example, we report the formation of RCC-adducts on PDGFRβ and the subsequent dysfunction of the tyrosine kinase receptor. Experimental studies carried out on apoE$-/-$ mice showed a strong inhibition of carbonyl stress in vessels correlated with a significant reduction of atherosclerotic lesions. The generation of new drugs sharing potent antioxidant and carbonyl scavenger properties represents a new therapeutic challenge in oxidative stress-associated diseases.

S5-05

Redox signalling: A key role in the legume-*Rhizobium* symbiosis?

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The symbiosis between leguminous plants and rhizobia leads to the formation of a new organ, the N_2-fixing root nodule. There is now compelling evidence that reactive oxygen and nitrogen species and glutathione are involved in the symbiotic process. Indeed, hydrogen peroxide (H_2O_2) can be observed in infected cells of young nodules during the *Medicago-Sinorhizobium meliloti* symbiosis. Moreover, a bacterial mutant over-expressing a constitutive catalase displayed a modified nodulation phenotype, clearly indicating that the presence of H_2O_2 is essential for optimal symbiosis development: the expression of several important bacterial genes may be regulated by H_2O_2. On the other hand, a direct detection of nitric oxide (NO) was performed; NO was localized in the microsymbiont containing cells. A transcriptomic analysis of NO-modulated genes revealed that the expression of genes involved in the maintainance of the nodule redox state (including those involved in glutathione synthesis) may be under NO control. NO also appears to regulate the expression of genes related to protein degradation or encoding transcription factors. Furthermore, NO was found to modify the activity of several enzymes of the glycolysis and of asparagine synthetase in *Medicago truncatula* nodules. Finally, the deficiency in glutathione (GSH) appeared to inhibit the formation of the root nodules. A transcript profiling analysis was performed using a cDNA-AFLP protocol and it appears that GSH may regulate the expression of plant defence genes. Based on these data, the possible role of redox signalling in regulation of legume nodule development and metabolism will be discussed.

S5-06

S-nitrosylation of peroxiredoxin II E promotes peroxynitrite-mediated tyrosine nitration

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S-nitrosylation is emerging as an important mechanism for the transduction of nitric oxide (NO) bioactivity. By using a proteomic approach involving two-dimensional gel electrophoresis and mass spectrometry we identified, for the first time, proteins undergoing S-nitrosylation *in vivo* and characterized the functional significance of this modification for PrxII E, a type II peroxiredoxin. Biochemical and genetic evidence indicate that PrxII E functions in detoxifying peroxynitrite anions ($ONOO^-$), a reactive nitrogen species generated from NO under oxidative stress that is able to nitrate and to oxidize biomolecules. S-nitrosylation abrogates PrxII E activities enhancing $ONOO^-$ derived tyrosine nitration, which is now becoming increasingly recognized as a post-translational protein modification that modulates catalytic activity and cell signalling. We concluded that NO regulates the effects of its own radicals through S-nitrosylation of crucial components of the antioxidant defense system that function as common triggers for reactive oxygen species (ROS) and NO mediated signalling events.

S5-07

Cellular redox metabolism, programmed cell death and defence responses in plants

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Photosynthetic metabolism involves complex interactions between different sub-cellular compartments. Chloroplasts and mitochondria play important roles in cellular redox metabolism connected to photosynthesis and respiration. Chloroplasts are important sources of reactive oxygen species (ROS). For example, hydrogen peroxide is produced both by pseudocyclic electron flow and during photo-respiratory glycolate metabolism. The mitochondrial electron transport chain oxidizes NADH produced during photo-respiratory and respiratory metabolism. To analyse the impact of redox metabolism on metabolic integration and oxidative signalling, we have used phenotypic, metabolic and transcriptomic analysis of tobacco knockout mutants for mitochondrial complex I. Aspects of these studies and key conclusions will be described. In particular, it will be shown (1) that light and ROS are not required for the execution of harpin-induced

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programme cell death and (2) that mitochondrial NAD status may be an important determinant not only of stress tolerance but plant phenotype through effects on the integration of carbon-nitrogen metabolism in leaves and associated signalling pathways.

SESSION VI—DIET AND ENERGY REGULATION OF CELL SIGNALLING

S6-01
Diet and regulation of cell signalling session: An overview

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α-Lipoic acid (LA) is an example *par excellence* of a dietary factor modulating oxidative stress, cell signalling and energy. In eukaryotes LA is synthesized in chloroplasts and present in mammalian tissue in minute amounts. LA in mitochondria acts as a cofactor of pyruvate dehy-drogenase (PDH) and other α-keto acid complexes and the glycine cleavage system. LA is reduced to dihydrolipoic acid (DHLA) by dihydrolipoamide dehydrogenase, the E3 enzyme of PDH, and by thioredoxin. LA increases cell GSH through Nrf2 activation and, consequently, high g-glutamylcysteine ligase levels (rate-controlling enzyme in GSH synthesis) [1] and also by DHLA recycling of cystine to cysteine [2]. In some systems LA and acetyl-L-carnitine (ALC) have synergistic effects. ALC facilitiates transport of long chain polyunsaturated fatty acids across membranes and B oxidation in mitochondria generates large amounts of NADH. By metabolism, both LA and ALC act complimentarily on NADH oxidation and reduction, respectively. In primary cortical neurons exposed to 4-hydroxy-nonenol (HNE)-induced oxidative stress RS-LA and ALC led to the activation of phosphoinositol-3 kinase (PI3K), PKG and ERK1/2 pathways, which play essential roles in neuronal cell survival [3]. This study demonstrated a cross-talk among the PI3K, PKG and ERK1/2 pathways in cortical neuronal cultures that contributes to LA and ALC-mediated pro-survival signalling mechanisms. In differentiated 3T3 adipocytes R-LA activates transcription factors and signalling pathways, stimulates fatty acid metabolism and results in a spectacular increase in mitochondrial Biogenesis [4]. R-LA and ALC act synergistically in a range of concentrations. The effects of R-LA on cell redox and metabolism appear to establish an energy–redox axis through activation of transcription factors and cell signalling cascades that increase the cell's reducing power and energy levels.

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S6-02
Modulation of selenium homeostasis by insulin and stressful stimuli via FoxO factors?

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Selenoprotein P (SeP) is the central selenium transporter in plasma, supplying extra-hepatic tissues with selenium. A transcriptional regulation of SeP production by the forkhead box transcription factor FoxO1a was demonstrated in reporter gene assays using the human SeP promoter as well as point and deletion mutants thereof, by RT-PCR and at the level of selenite-induced release of SeP from human hepatoma cells. Insulin, stimulating the phosphorylation and inactivation of FoxO1a via phosphoinositide 3-kinase (PI3K) and the Ser/Thr-kinase Akt, suppressed SeP release from hepatoma cells. This suppressive effect of insulin on SeP expression was attenuated by inhibitors of PI3K. Hence, we propose that selenium homeostasis is modulated by insulin signalling via hepatic SeP expression being regulated by FoxO1a. Insulin-like signalling is stimulated by stressful stimuli, including ROS, UV radiation and heavy metal ions. Exposure of human and rat hepatoma cells to Cu(II) or Zn(II) resulted in a PI3K/Akt-dependent phosphorylation, inactivation and nuclear exclusion of transcription factors of the FoxO family as well as an impairment of FoxO target gene expression. In summary, the SeP gene is a novel target of the PI3K/Akt/FoxO cascade. Due to the prominent role of SeP in selenium transport, regulation of SeP expression by FoxO1a establishes a novel link between insulin signalling and selenium homeostasis. Moreover, exposure of cells to stressful stimuli imitates insulin signalling, suggesting a link between cell stress and selenium homeostasis.

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S6-03
Nutritional modulation of sirtuin expression is mediated by redox changes

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Sirtuins are NAD-dependent deacetylases that modulate metabolism, the rate of ageing and other critical physiological parameters. Thus, metabolic regulation of these enzymes is of critical importance. Nicotinomide is known to inhibit the enzyme. The role of the NAD/NADH redox ratio in the regulation of sirtuins has also been proposed. To test this idea, researchers have measured NAD and NADH in several cellular models. However, in a pioneer study, Krebs and co-workers showed that 'direct measurement of tissue content of NAD and NADH do not supply the required information: they fail to differentiate between free and bound nucleotides and they give no information on the distribution of the nucleotides between the various cell components which is known to be uneven'. This difficulty can be overcome by measuring the concentration of the oxidized and reduced metabolites of suitable NAD-linked dehydrogenases. We have used cell culture, invertebrates (Drosophila) and vertebrates (mice) models to study the regulation of sirtuins. We have found that buffering the NAD/NADH pair with known concentrations of lactate and pyruvate regulates sirtuin expression in fibroblasts in culture. Ethanol, which affects the NAD/NADH ratio, also up-regulates sirtuin expression. In Drosophila, ethanol also up-regulates sirtuin expression and this results in an increased average life span of the animals. NAD/NADH ratio also regulates sirtuin expression in vertebrates. Exercise causes a significant increase in lactate-pyruvate ratio and thus changes in NAD-NADH. This results in an increase in the expression of sirtuins and in an increased life span. The importance of this study lies in the fact that sirtuin expression does not depend on levels of free NAD or NADH but rather on the ratio of these co-enzymes in tissues. This ratio can be modulated by many physiological manipulations like exercise or moderate ethanol intake. We have observed that these metabolic changes result in significant increases in average life span of the animals. The importance of these facts to understand the role of oxidation in longevity will be fully discussed.

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S6-04
The concept of oxidative stress in pathology

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Helmut Sies coined in 1985 the concept of oxidative stress as a situation of imbalance with an excess of oxidants or a decrease of antioxidants that equally lead to cell damage. The concept was immediately accepted and widely utilized to plan and explain experimental research. After 20 years the concept keeps its original strength, validity and applicability. Recently, a difference started to be made between oxidative stress as a reversible situation and oxidative damage as an

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irreversible situation, but the limit is not clear. The difference is similar to the one between reversible and irreversible cell injury in classic cell pathology. The objective is to link, phenomenologically and kinetically, the biochemical events that lead from oxidative stress to cell death, either by apoptosis or by necrosis, with the morphological evidence of histochemistry and electron microscopy. Another difference is also made between cellular oxidative stress and systemic oxidative stress. In the latter case, the oxidative stress markers are determined in blood and plasma (TBARS, TRAP and t-butyl-hydroperoxide initiated chemiluminescence). These markers indicate both free-radical mediated chain reactions in the vascular space and oxidative damage occurring in target organs. Phagocyte produced oxidants or lipid hydroperoxides absorbed from food provide the reactants for the initiation reactions in the vascular space. Neurological diseases, such as Parkinson's and Alzheimer's diseases and vascular dementia, are associated to systemic oxidative stress with increased plasma levels of the mentioned markers.

S6-05

(−)−Epicatechin 3′-O-Methyl Ether and Related Compounds as Inhibitor of Endothelial NADPH Oxidase: On the Mechanism of Elevation of Bioavailable NO by Dietary Flavanols

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Dietary flavanols, e.g. from the procyanidins in flavanol-rich cocoa, are known to improve endothelial function as demonstrated by an increased flow-mediated dilation (FMD). This effect is ascribed to a concomitant increase in circulating bioavailable NO, measured as RNO in plasma, leading to increased cGMP in vascular smooth muscle. The mechanism of this acute response operates through inhibition of the loss of NO rather than though an increased generation rate of NO. Based on experiments with human umbilical vein endothelial cells (HUVEC) and NO imaging analysis, we conclude that the elevation of RNO is due to inhibition of endothelial NADPH oxidase. Shutting down superoxide production will lower the loss of NO in the reaction forming peroxynitrite, leaving more of the NO available for diffusion to the smooth muscle cell to elevate cGMP levels. Thus, this mechanism would not require a short-term increase in flux through endothelial NO synthase (eNOS). HUVEC express catechol O-methyl transferase (COMT), and an inhibitor of COMT blocks the epicatechin effect. The IC(50) for inhibition of NADPH oxidation of these flavanol metabolites is substantially lower than that of the standard NADPH oxidase inhibitor, apocynin.

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YOUNG INVESTIGATOR SESSION—SFRRE WINNERS

YIL-01

The role of HO-1 in the formation of oxidized stress induced by ALA-PDT

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Photodynamic therapy (PDT) is an established anti-tumour therapy utilizing the photogeneration of reactive oxygen species (ROS). PDT is carried out using 5-aminolevulinic acid (ALA) which is converted to the natural porphyrin PPIX by the endogenous heme synthesis pathway. PPIX is an excellent photosensitizer and in cancer cells highly accumulated due to a decreased activity of ferrochelatase. The formed ROS can damage certain cell components by oxidative processes, which may result in cell death. Unfortunately, the effects of ALA-PDT are *in vivo* often superficial which limits its application in anti-cancer treatment. Additionally it is assumed that induction of HO-1 acts as a protector against oxidative stress by the generation of biliverdin, Fe and CO. Therefore, our study has attempted to investigate the combination of PDT with inhibitors of HO-1 in order to enhance the efficacy of ALA-PDT treatment. In a first series of experiments melanoma cells (WM451) were loaded with ALA and exposed to non-thermal illumination (420–800 nm) with doses between 0.54–5.40 J/cm^2. It was shown that the formation of ROS (DCF-assay) and oxidative stress markers increased in a dose-dependent manner. Similarly, induction of HO-1 was shown by qPCR and immunoblot. Interestingly, inhibition of HO-1 by ZnPPiX decreased viability of the cells. The formation of ROS and oxidation of cellular macromolecules was also determined. The results did show that the anti-tumour effects of PDT may be enhanced by the simultaneous application of HO-1 inhibitors, which has now to be proven *in vivo*.

YIL-02

Vitamin C and nitrite react in human erythrocytes to give a novel product, Asc_2NO

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Nitrite ions (NO_2^-) comprise the largest vascular storage pool of nitric oxide (NO). Until recently, central dogma of NO metabolism considered nitrite to be an end, even waste product of NO biosynthesis. However, the latest results proved that NO_2^- is important in recycling of nitric oxide and several mechanisms have been proposed to explain this reaction. Vitamin C (ascorbate) is known to enhance NO production from acidified nitrite. In our previous study we showed that ascorbate and nitrite react at neutral pH to yield a novel product (Asc_2NO), which releases NO under physiologically relevant conditions. It is well known that erythrocytes (RBC) can accumulate high levels of ascorbate exhibiting a peculiar property as a sink of nitrite as well. Our hypothesis was that in erythrocytes nitrite reacts with ascorbate to give Asc_2NO. To prove it we developed indirect method for Asc_2NO detection based on detection of Cu^{2+}-catalysed NO release from the product. Here we provide evidence that Asc_2NO may be formed *in vivo* in both untreated and nitrite or/and ascorbate-supplemented human erythrocytes. We found basal level of Asc_2NO (7 ± 3 μM) to increase several fold when humans were subjected to 2-weeks of vitamin C supplementation (19 ± 6 μM). Asc_2NO formation was also enhanced when RBC were treated with nitrite and/or ascorbate. Furthermore, our results showed that Asc_2NO can S-nitrosylate protein thiols, increasing formation of S-nitrosohaemoglobin. These findings shed a new light on vitamin C/nitrite metabolism, forming the basis for a novel approach to understand numerous effects of NO signalling pathways.

YIL-03

Genetic polymorphisms controlling oxidative stress influence the activity of rheumatoid arthritis

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Reactive oxygen (ROS) and nitrogen (RNS) species are involved in the pathology of rheumatoid arthritis (RA) as they are generated in synovial fluid of inflamed joints. RA activity may be modified by genetic polymorphisms of ROS detoxifying enzymes such as manganese superoxide dismutase (MnSOD), extracellular superoxide dismutase (EC-SOD) and catalase (CAT) as well as polymorphisms of factors that stimulate ROS and RNS production such as tumour necrosis factor-alpha (TNFa) and inducible NO synthase (iNOS). The aim of our study was to determine if these polymorphisms influence the activity of RA. Genotyping approach was used to determine MnSOD Ala-9Val, EC-SOD Arg213Gly, CAT C-262T, TNFa G-308A and iNOS (CCTTT)n promoter polymorphisms in 210 RA patients. Disease activity was assessed by disease activity score of 28 joint counts (DAS28). Multiple linear regression analysis was used to define the influence of polymorphisms on the disease activity. No significant association between EC-SOD Arg213Gly, CAT C-262T, iNOS (CCTTT)n polymorphisms and disease activity was observed. However, patients with MnSOD-9Val/Val genotype had significantly higher mean DAS28 values than carriers of -9Ala-encoding allele ($B = 0.291$, $P = 0.036$). On the contrary, carriers of at least one TNFa-308A allele had significantly lower mean DAS28 values that patients with TNFa-308GG genotype ($B = -0.349$, $P = 0.007$). Furthermore, carriers of both MnSOD-9Ala-encoding allele and TNFa-308A allele had lower mean DAS28 values than patients with MnSOD-9Val/Val and TNFa-308GG genotype ($B = -0.395$, $P = 0.009$). Our results suggest that TNFa G-308A and MnSOD Ala-9Val polymorphisms alone and in combination influence RA activity.

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YIL-04

Proteasome activity deregulation in LEC rat hepatitis: Following the insights of transcriptomic analysis

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LEC rats have a mutation in a gene related to liver copper excretion, the Atp7b gene. As a consequence, this rat strain shows an abnormal copper accumulation in the liver. The copper accumulation accelerates Reactive Oxygen Species (ROS) production. This is believed to be the origin of the acute hepatitis and the subsequent hepatocellular carcinoma that spontaneously develop in these rats. Here we present a microarray analysis of LEC rats at different stages of hepatitis compared against D-penicillamine-treated LEC rats. Multivariate statistical analyses as Partial Least Square (PLS) regression between transcriptomic and hepatitis markers in plasma lead us to the selection of genes related to hepatitis development. Gene ontology classification of selected genes shows a deregulation in protein metabolism-related genes. Genes from proteasome pathway were up-regulated; however, 20S proteasome activity was diminished during hepatitis. One mechanism of proteasome inactivation is oxidative stress. Hence, we propose a deregulation of proteasome genes face to oxidative inactivation of proteasome activity during hepatitis in LEC rats. Our results add to the known oxidative stress and inflammation characterization of the hepatitis process, leading to new insight concerning hepatitis and hepatocarcinogenesis development.

YIL-05

Fit at old age—adaptation or selection?

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When animals grow and age, they change morphologically and biochemically. In many cases this leads to a change in behavioural and physiological response towards environmental stressors. These stressors may either be abiotic like temperature or oxygen availability, but can also be biotic stressors like predation or competition. Bivalves can be used as model organisms to investigate the effect of physiological ageing on stress tolerance, as the shells exhibit age rings like trees and thus individual age of each animal taken from different environments can be determined. Different aged individuals of more active (scallops) and more passive (mud clams) bivalve species were exposed to environmental stress factors these species experience in the specific environments: mud clams were exposed to anoxia and hypoxia and scallops to predation. Parameters of redox ratio, energy charge, antioxidant capacity, free radical damage and aerobic and anaerobic energy generation were investigated in specific tissues of control and experimental individuals. Marked differences were found in general physiological state and behaviour and physiological response towards the different stressors, between younger and older bivalves. In many cases behaviour could be explained by physiological and morphological properties. Younger individuals showed in general a more pronounced cellular response and higher mortality compared to the older individuals. The question arises if the less pronounced stress response and vulnerability in older/bigger individuals is due to adaptation of selection, i.e. have old aged individuals always been fitter compared to other individuals of the same cohort or did their physiological fitness change with age?

YIL-06

The isolated mature skeletal muscle fibre: A model system to study intracellular ROS generation

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Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are constantly produced by skeletal muscle and play an important part in signalling adaptive responses to contractile activity. To elucidate the roles of ROS/RNS in detail it is essential to understand the sites and mechanisms of their generation in skeletal muscle. This is particularly important for the intracellular production, since few detailed studies of these processes have been undertaken. The aim of this study was to examine the real-time generation of intracellular ROS and RNS in resting and electrically stimulated isolated mature mouse skeletal muscle fibres. The merit of this preparation is that the muscle fibres are mature in comparison with immortalized or primary myoblasts in culture. In addition, the system allows the analysis of ROS and RNS generation in the absence of non-myogenic cells that may contribute to ROS/RNS production. Single muscle fibres were isolated from the *Flexor Digitorum Brevis* muscles of mice and intracellular ROS/RNS generation was detected with the use of three fluorescent probes, DCFH, DAF-FM and hydroethidine. Fibres were loaded with the appropriate probes and subjected to resting or contracting protocols and their intracellular ROS/RNS generation was analysed by fluorescence microscopy. Fibres subjected to electrical stimulation displayed increases in DCF, DAF-FM and ethidium fluorescence compared with rested fibres. These rises in intracellular fluorescence were abolished by respective treatments with an antioxidant, NOS inhibitor and a superoxide scavenger. This data indicates that intracellular generation of ROS and RNS are increased during periods of muscle contraction. This work describes a novel technique that will provide a valuable tool for measuring the real-time generation of intracellular ROS in isolated muscle fibres and will improve knowledge of the roles of ROS in muscle function.

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YIL-07

Infrared A radiation triggers retrograde mitochondrial signalling to modulate gene expression

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Infrared-A-radiation (IRA; 760–1440 nm) is a major component of natural sunlight. We have previously shown that IRA radiation is a potent inducer of MMP-1 expression in human dermal fibroblasts. Since proteins of the mitochondrial electron transport chain (mtETC) absorbs IRA and oxidative stress is known to modulate gene expression, we asked whether IRA leads to the formation of mitochondrial ROS and, if yes, whether this would be of functional relevance for IRA-induced signal transduction, its subsequent gene expression and the resulting ageing processes. Experiments utilizing Mitosox, a dye specific for mitochondrial superoxide radical anions, revealed that IRA at physiologically relevant doses leads to an at least 3-fold increase in mitochondrial superoxide. We have found that IRA induced MMP-1 expression in primary human fibroblasts can be abrogated by using mtETC inhibitors. This effect was specific since MMP-1 expression induced by other wavelengths (i.e. UVA and UVB) was not inhibited. In addition we were able to demonstrate that mtDNA depleted (rho−) cells do not show IRA-induced MMP-1 upregulation in contrast to corresponding rho+ cells. UVA and UVB induced MMP-1 induction was not altered by absence of mitochondria. Also, increasing the amount of mtETC compounds by overexpression of a stimulator of mitogenesis, PCG-1, led to an increased sensitivity towards IRA, where effects of UVA and UVB were unaltered towards the control transfected cells. Taken together our studies demonstrate that IRA induced gene expression involves retrograde mitochondrial signalling, mediated by ROS leaking from the respiratory chain.

POSTERS

P-001

Evaluation of oxidative stress and hematological parameters in professional cyclists after exhaustive exercise

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It is well established that intensive exercise is associated with an increased formation of reactive oxygen species (ROS) and impaired antioxidant defenses. These effects induce an increase in glutathione oxidation, oxidative protein changes and cell membranes damage as a result of lipid peroxidation. In the present study we purposed to determine the effects of exercise 3 h after finishing flat cyclist stage. The activity/levels of various oxidative stress markers were evaluated in plasma and erythrocytes: glutathione reductase (GR), glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), oxidized glutathione (GSSG), thiobarbituric acid reactive substances (TBARS), as well as a number of haematological parameters: red and white blood cells (RBC and WBC) and sub-types, reticulocytes, platelets, haemoglobin (Hb), haematocrit (HCT), mean cell volume (MCV), mean cell haemoglobin (MCH) and mean cell haemoglobin concentration (MCHC). In recovery time (3 h after exercise), the CAT and GR activities were significantly increased in erythrocytes, whereas the GPx activity decreased. Likewise, the TBARS and GSH levels decreased significantly in both plasma and erythrocytes in comparison with controls values. However, GSSG only increased in erythrocytes. An increase in RBC, WBC with neutrophilia and lymphopenia and reticulocytes was also observed. These findings show that exhaustive exercise caused oxidative stress and alterations in haematological parameters, 3 h after having concluded the cyclist race.

P-002

Effects of sulphasalazine treatment on male rats' reproduction

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Sulphasalazine (SASP) has been reported to cause infertility in men. The aim of this study was to evaluate the endogenous antioxidant capacity and oxidative damage in reproductive male tissues. We suggested an oxidative stress-like mechanism inductor of infertility. Adult male Sprague-Dawley rats (10/group) were orally administered 0 or 600 mg SASP/kg body weight for 14 days followed by 2 weeks without treatment. Testicular and epididymal weights and histopatological study were evaluated, as well as concentration, motion and morphology sperm. Oxidative stress biomarkers were also determined in testes and epididymus. No changes were seen in testicular or epididymal weights. Spermatid number in testes and epididymus decreased by SASP administration, but only significantly in testes. After 14 days without administration, spermatid number in epididymus was significantly increased. In addition, spermatozoa morphological abnormalities (amorphous spermatozoa head and spermatozoa without tail) and a decrease in some types of motility (high and moderate) was also observed in rats treated with SASP. Histopathological examination of the testes revealed tubular atrophy in treated groups. In rat testes, SASP administration induced a significant decrease of glutathione reductasa (GR) and reduced glutathione (GSH). Also a decrease in thiobarbituric acid-reactive substances (TBARS) was observed in animals treated with SASP for 14 days. The results of oxidative stress markers in epididimus showed a significant decrease in glutathione peroxidase activity (GPx) and a significant increase of GSH. The results of this study suggest that SASP induce alterations in several seminal parameters, histological changes and oxidative stress in testes and epididimus.

P-003

Inhibitory effects of different antioxidants on hyaluronan depolymerization

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Three sodium hyaluronan (HA) non-cross-linked commercial available products (Condrox, Hyalgan and Suplasyn), which differ in molecular weight and animal or non-animal source, were depolymerized by hydroxyl radicals generated from hydrogen peroxide and cupric ions. Inhibition of HA degradation by four well-known antioxidants was investigated, as HA can scavenge reactive oxygen species (ROS). Change in hyaluronan molecular weight was observed by size exclusion chromatography. Inhibition of HA degradation was estimated from the retention times observed. It was found that HA degradation was inhibited in a clearly concentration-dependent manner by mannitol, thiourea and vinpocetine. Propofol also inhibited the depolymerization, but its concentration-dependent effect was not so clear. The antioxidant concentrations at which HA degradation was decreased by 50% were lower than 5 μM for vinpocetine in the HA studied, whereas for the other three antioxidants studied these concentrations were higher. Furthermore, the maximum inhibition percentages were higher than 50% for vinpocetine, unlike the percentages for mannitol, thiourea and propofol. Although many factors are involved in a therapeutic response, the results obtained in this study support the idea that HA may be protected from ROS attack by the concomitant use of well-known antioxidants.

P-004

Failure in mitochondrial biogenesis causes mtdna depletion in secondary biliary cirrhosis in rats

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A loss of mitochondrial function characterizes chronic colestasis [1] and its consequences are lower ATP synthesis [2] and increased generation of reactive oxygen species [3]. Mitochondrial biogenesis is driven by coordinate action of transcription factors that act within the nucleus and the mitochondria. The aim was to assess the regulation of mitochondrial biogenesis in the liver during chronic cholestasis in rats. Three groups of rats (BDL, bile duct ligated; SHAM, control; PAIR-FED to BDL animals) were employed for the study. Livers were collected at day 28 and analysed. PCG-1alpha, NRF-1, Tfam transcription factors were measured by RT-PCR and western blotting. GABP, mitochondrial chaperones Hsp70 and Hsp60, and cytochrome c were measured by western blotting; mitochondrial-DNA/nuclear-DNA ratio (mtDNA/nDNA) was also measured by RT-PCR. Protein levels of PGC-1alpha did not change but GABP and Tfam were decreased in BDL. NRF-1 was completely phosphorylated in BDL animals. mRNA of PGC-1alpha and NRF-1 were increased and Tfam was markedly decreased in BDL. Cytochrome c in mitochondrial preparations was increased but mtDNA/nDNA was decreased in BDL. In conclusion, a decrease in the protein levels of the transcription factors that promote mitochondrial biogenesis occurred in BDL animals. Compensatory mechanisms, such as NRF-1 phosphorylation and increased Hsp70, promote enrichment in nuclear encoded proteins in the mitochondria. In contrast, down-regulation of Tfam leads to depletion of mitochondrial DNA in liver of BDL rats.

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P-005

Modulation of p53 gene expression and caspase-3 activity by γ-glutamylcysteine ethyl ester in kainic acid model of neurodegeneration

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The tripeptide glutathione is very important for the cellular defence against reactive oxygen species including non-organic molecules such as the superoxide anion, hydrogen peroxide and hydroxyl radicals. An imbalance of GSH is observed in a wide range of pathologies, including neurodegenerative disorders, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, stroke and epilepsy. The understanding of the molecular pathophysiology of these brain dysfunctions will provide new clues that may lead to effective therapeutic approaches that will limit damage and slow cells. Recent studies showed that γ-glutamylcysteine ethyl ester (GCEE) has the ability to increase glutathione

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(GSH) levels in rat brain and displays antioxidant activity similar to GSH, as asssessed by various *in vitro* indices such as hydroxyl radical scavenging and dichlorofluroescein fluorescence. Kainic acid (KA) is an excitotoxic amino acid which is used to develop a rat model of limbic-cortical neuronal damage. It causes calcium influx in the brain, which, in turn, generates ROS. There is evidence that generation of reactive oxygen species and induction of an apoptotic pathway are involved in the mechanism of KA-induced neuronal damage. Although antioxidant properties of GCEE have been demonstrated, relatively little is known about their regulator effect on gene expression. In our study, GCEE modulates the expression levels of p53 gene in rat brain following KA-induced seizures. In addition GCEE significantly increases the caspase-3 enzyme activity in brain against KA treatment. Therefore, we conclude that the regulation of apoptotic/anti-apoptotic mechanisms by GCEE may indicate its potential neuroprotective effect in KA-mediated neurodegeneration.

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P-006

Permeability and localization of nitroxide pre-fluorescent probes in epithelial cells

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The high sensitivity of nitroxide pre-fluorescent probes has allowed one to evaluate hydrogen transfer rate reactions from highly reactive phenols towards nitroxide radicals and also to detect free radical species in chemical systems. In the present work, we studied the permeability of the nitroxide pre-fluorescent probes QT and $C_{314}T$ in epithelial cells by confocal microscopy. The experiments *in vivo* showed efficient permeability across the plasma membrane and a fluorescence increase on time, according with a fast reaction with cytosolic ascorbic acid. At high nitroxide probe concentrations incorporation of the probes was observed into the nucleus with intercalation into the DNA. This is in agreement with membrane instability due to an apoptotic response of the cell. This conclusion is supported by the positive detection of apoptosis markers, caspases-3 activation and rhodamine labelling of DNA breaks, observed in the presence of high concentrations of the nitroxide probes in comparison with the isolated chromophore.

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P-007

The pathways of cell death in cardiomyocytes induced by vanadate

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Necrosis and apoptosis are the most often reported cell death pathways. Vanadium is an element well known for its toxicity, but is also considered to be essential for many organisms. This study investigates the effects of two vanadate solutions in the pathways of cardiac myocytes cell death. Cells exposed to 10 μM vanadate (IC_{50}) did not show signs of apoptosis, such as caspase 3-activation. On contrary, a 40% decrease of ATP content, characteristic of necrotic cell death, was observed 24 h after vanadate administration. Vanadate treatment increased intracellular Ca^{2+} level from 60 nM to 240 nM, as observed with Fura-2 probe. In necrosis, this effect would induce an increase of reactive oxygen species production. However, a 70% decrease of mitochondria superoxide anion generation was observed upon exposition to vanadate. In mitochondria, known to play an important role in cell death, vanadate induces membrane depolarization, as observed by the fluorescent probe TMRE, at concentrations as low as 7 μM. In isolated mitochondria, it was also observed that low vanadate concentrations inhibit oxygen consumption (oxygen electrode) and induce membrane depolarization, as observed with the fluorescent probe JC-1. Putting it all together, it is suggested that vanadate, an element with a versatile role in organisms, induce a necrotic cell death in cardiac myocytes. The pathway of cardiac cell death induced by vanadate is associated with a large impairment of mitochondrial bioenergetics, but also with an altered Ca^{2+} homeostasis and decreased intracellular oxidative stress.

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P-008

PON1 activity in pre-menopausal and post-menopausal women

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Human paraoxonase 1 (PON1), encoded by *PON1* on chromosome 7q21.3, is a serum arylesterase that is associated with high-density lipoprotein-apolipoprotein A-I (HDL-apoA-I). HDL-bound PON1 protects low-density lipoprotein (LDL) from oxidation, probably as a result of its ability to hydrolyse specific oxidized lipids. The common coding polymorphism Q192R has been described to influence PON1 activity. This study was performed in women under two different hormonal statuses (pre-menopausal and post-menopausal women). The aim of this work was (1) to determine the relationship between PON1 activity and serum HDL-cholesterol (HDLc)- and apoA-I levels and (2) to assess for differences in PON1 activity according to Q192R polymorphism between both groups. HDLc was quantified by commercially available enzymatic kits and apoA-I by immunonephelometry. PON1 activity was measured spectrophotometrically, using paraoxon (paraoxonase activity) or phenylacetate (arylesterase activity) as substrates. Genotyping was performed by the polymerase chain reaction-restriction fragment length polymorphism assay. PON1 arylesterase activity positively correlated with serum apoA-I and HDLc levels only in pre-menopausal women. However, PON1 paraoxonase activity did not correlate with HDLc or apoA-I. Paraoxonase activity was genotype-associated according to the order RR > QR > QQ. In contrast, R allele carriers showed lower arylesterase activity in the studied populations. No differences were detected in the PON1 activity according to the hormonal status.

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P-009

Serum redox alterations during *in vitro* fertilization therapy: Involvement of COMT Val108/158Met polymorphism

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During *in vitro* fertilization (IVF) therapy women undergo changes in the plasma 17β-estradiol levels from undetectable (E2min) to supra-physiological (E2max) values. Catechol-*O*-methyltransferase (COMT) catalyses the *O*-methylation of catecholestrogens, thus preventing them from entering in redox cycles. The aim of the present work was to determine whether these E2 changes were associated with alterations in the serum antioxidant status depending on the *COMT* Val108/158Met polymorphism. Sera at E2min and E2max stages from 157 women undergoing IVF were selected. Samples were subjected to *in vitro* Cu^{2+} oxidation and absorbances were registered at 245 and 268 nm. Total

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antioxidant activity (TAA) was measured by the $ABTS^{+\cdot}$ decolourization method. Serum α- and γ-tocopherol and malondialdehyde (MDA) were determined by HPLC. *COMT* Val108/158Met polymorphism was detected by tetraprimer ARMS-PCR. Specifically the *COMT* high activity allele (Val) was associated with a decreased serum antioxidant status at E2max respecting E2min, expressed by: (a) increased serum oxidation rates in the lag- and propagation phases, and a decreased lag time; (b) decreased TAA; (c) reduced α- and γ-tocopherol content; and (d) increased MDA. We conclude that the *COMT* low activity genotype (Met/Met) confers antioxidant protection respecting the high activity allele during the ovarian stimulation achieved in IVF.

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P-010

Protection of anthocyanins against human LDL oxidation and their structure–activity relationship: A key component in the French paradox

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An increased interest in anthocyanins and their biological effects has emerged in the last years. They are a sub-group of flavonoids responsible for the colour and most of the benefits of moderate consumption of red wine. The present study was designed to evaluate and compare the antioxidative properties of four structurally related anthocyanins—pelargonidin, cyanidin, malvidin and malvidin-3-glucoside—against human LDL oxidation promoted either by AAPH-generated peroxyl radicals, or two physiologically relevant oxidants, ferrylmyoglobin and peroxynitrite. Their ability to recycle α-tocopherol (α-TOH), the most abundant LDL-lipophilic antioxidant, was also studied. When LDL oxidation was initiated either by AAPH or ferrylmyoglobin, as determined by the fluorescence decay of incorporated *cis*-parinaric acid and conjugated dienes formation, those anthocyanins strongly inhibit LDL oxidative damage, cyanidin and malvidin being far more efficient as compared with pelargonidin. Also, malvidin-3-glucoside exhibited a stronger antioxidant activity than malvidin, the non-glycosilated derivative. Peroxynitrite-promoted LDL apoprotein modifications, as evaluated by apoB net surface charge alterations, were efficiently inhibited by cyanidin, malvidin or malvidin-3-glucoside, while almost no effects were observed with pelargonidin. Moreover, all the anthocyanins significantly decreased peroxynitrite-mediated carbonyl groups formation in LDL. EPR measurements of α-tocopheroxyl radical showed that the anthocyanins strongly reduce the signal intensity of that radical pointing to their highest abilities to recycle α-TOH, although malvidin-3-glucoside was far less effective. Our results corroborate the relevance of patterns of hydroxyl or methoxyl substitution and glycosylation to the modulation of antioxidant activities of anthocyanins. Also, they suggest that the consumption of anthocyanins through the intake of red wine may greatly contribute to protect LDL from oxidative damage and, therefore, may be a key component in the French paradox.

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P-011

Antioxidant status of human follicular fluid: Implications in female infertility

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The aim of this work was to determine the antioxidant status in follicular fluid and assess its involvement in woman infertility. ORAC (oxygen radical absorbance capacity) and TAC (total antioxidant capacity) were measured in follicular fluid aspirated from follicles during oocyte pickup from women enrolled in IVF therapy ($n = 30$) and were compared with the activities in follicular fluid aspirated from healthy control donors ($n = 30$). ORAC was measured by assessing the area under the fluorescence decay curve (AUC) of fluorescein with AAPH as free radical initiator in the presence of the sample as compared to that in the blank in which no antioxidant is present. The ORAC value was also determined in the soluble fraction after acetone deproteinization. TAC was measured by the $ABTS^{+}$ radical cation decolourization method. The follicular fluid of subfertile women exhibited a significant lower ORAC value compared with control donors (5783 ± 1237 vs 6492 ± 1066 μM Trolox, $p = 0.021$). No differences in either the ORAC value in deproteinized samples or TAC were found between both groups. In conclusion, the reduced antioxidant activity in the follicular fluid suggests a role for free radicals in women infertility, probably contributing to impairment of reproduction in these patients.

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P-012

Free radical scavenging activity of different almond (*Prunus dulcis*) varieties

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Reactive oxygen species are known to be implicated in many cell disorders and in the development of many diseases including cardiovascular diseases, atherosclerosis, cataracts, chronic inflammation or neurodegenerative diseases such as Alzheimer's or Parkinson's disease. Thus, synthetic antioxidants are widely used in the food industry, but, because of their toxic and carcinogenic effects, their use is being restricted. The pursuit for novel natural sources of bioactive compounds, namely those who present antioxidant activity, has been acquiring higher significance, since these compounds may contribute to the prevention of diseases in which free radicals are implicated. In this study, the antioxidant properties of different almond varieties (*Casanova*, *Duro Italiano*, *Ferraduel*, *Ferranhês*, *Ferred Star*, *Guara*, *Molar*, *Orelha de Mula* and *Pegarinhos*) were evaluated through several biochemical assays: DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity, reducing power, inhibition of β-carotene bleaching, inhibition of oxidative haemolysis in erythrocytes, induced by 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) and inhibition of lipid peroxidation in pig brain tissue through formation of thiobarbituric acid reactive substances (TBARS). For all the methods, EC_{50} values were calculated in order to evaluate the antioxidant efficiency of each variety. The total phenols and flavonoid contents were also obtained and correlated with antioxidant activity. *Ferred Star* and *Duro Italiano* revealed better antioxidant properties, presenting lower EC_{50} values, particularly for lipid peroxidation inhibition in TBARS assay. The highest antioxidant contents (phenols and flavonoids) were also found for these varieties.

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P-013

Free radical scavenging activity and bioactive compounds of five *Agaricus* sp. edible mushrooms

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Reactive oxygen and free radicals play an important role in cellular injury and the ageing process and also are considered to induce the lipid peroxidation that causes the deterioration of foods. Although organisms have endogenous antioxidant defences produced during normal cell aerobic respiration against the reactive oxygen species, other antioxidants are taken from the diet, both from natural or synthetic origin. Thus, synthetic antioxidants are widely used in food industry, but because of their toxic and carcinogenic effects, their use is being restricted. Individual tocopherol profile of five *Agaricus* mushroom species, widely consumed in Portugal, was obtained by high performance liquid chromatography coupled to a fluorescence detector

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(HPLC/fluorescence). Bioactive compounds such as phenols, flavonoids, ascorbic acid, β-carotene and lycopene were also determined. The lipid peroxidation inhibition capacity of the edible mushrooms was accessed by biochemical assays used as models for the lipid peroxidation damage in biomembranes, namelly inhibition of β-carotene bleaching in the presence of linoleic acid radicals, inhibition of erythrocytes haemolysis mediated by peroxyl radicals and inhibition of thiobarbituric acid reactive substances (TBARS) formation in brain cells. Their antioxidant properties were also evaluated through the reducing power determination and radical scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals. *A. silvaticus* revealed better antioxidant properties (lower EC_{50} values) than the other *Agaricus* species (*A. arvensis*, *A. bisporus*, *A. romagnesii*, *A. silvicola*) which is in agreement with the higher content of bioactive compounds found in the first specie. α-tocopherol and β-tocopherol were found in the samples, while γ- and δ-tocopherols were not detected.

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P-014

Effects of two new di(hetero)arylamines on prevention of oxidative stress induced in two different biological models

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We investigated the antioxidant properties of two new diarylamines from organic synthesis (MJQ1 and MJQ2), whose basic structure is similar to others, with reported antioxidant capacity, assessed by chemical tests, and biological activity against microorganisms. In this study we induced lipid peroxidation, in isolated rat liver mitochondria with ADP/Fe^{2+} and the diarylamine effects were examined by oxygen consumption and by TBARS method. The anti-peroxidative effect was maximal for MJQ1 at 50 nM (higher than the one reported for trolox) and for MJQ2 at 60 μM. At these same concentrations none of them depressed the transmembrane potential ($\Delta\Psi$) developed by mitochondria, neither the RCR nor the ADP/O ratio values. For 2-fold concentrations both diarylamines were effective in the prevention of mitochondrial $\Delta\Psi$ collapse observed on respiring mitochondria, with the TPP^+ electrode, which means a stabilization action on mitochondrial inner membrane. The results obtained were confirmed in whole cells. The compounds did not show toxicity to the L929 cell line, evaluated by the MTT reducing test and clearly protected from lipid peroxidation, induced by the oxidant pair ascorbate/Fe^{2+}, to the PC12 cell model, at the concentrations where maximal antioxidant effect was observed in mitochondria. The new diarylamines were revealed as very good antioxidants at very low concentrations, both in mitochondria and in whole cells. The results suggest a specific action site, for MJQ2, at mitochondrial complex I level. We are further exploring other intracellular targets for these new compounds that seem very promising against pathologies where oxidative stress is involved.

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P-015

Nitric oxide regulates Foxo3a activity to modulate mitochondrial ROS production

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It has long been established that, depending on the biological context, nitric oxide (NO) can play either a pro-oxidant or an anti-oxidant role; however, the underlying molecular mechanisms have only recently begun to be elucidated. Our studies have shown that NO can both positively and negatively regulate the expression of the main proteins involved in mitochondrial ROS detoxification. This regulation involves NO-mediated modulation of the mRNA levels of the transcriptional coactivator PGC-1α via the activation of the sGC/PKG pathway [1]. PGC-1α is the master transcriptional regulator of mitochondrial function and ROS protection systems [2]. Our current work focuses on understanding the mechanism that mediates NO-induced down-regulation of PGC-1α expression in the vascular endothelium. Since some NO effects are known to be mediated via activation of the PI3K/AKT pathway, we have examined the effect of inhibiting PI3K. Pre-treatment of primary endothelial cells with PI3K inhibitors prevented down-regulation of PGC-1α expression in response to the NO donor DETA-NO or the PKG activator 8-Br-GMPc, indicating that PI3K acts downstream of PKG. Similarly, pre-treatment with the AKT inhibitor AKT IV blocked NO-induced PGC-1α down-regulation, whereas DETA-NO increased the phosphorylation (activation) of AKT, supporting a role for the PI3K/AKT pathway in NO regulation of PGC-1α expression. Point mutation in a functional IRS box of the mouse PGC-1α promoter cancelled NO activity, suggesting the involvement of a FOXO transcription factor as a mediator of NO action. Importantly, FOXO factors are phosphorylated and inactivated by AKT. In further experiments we have shown that treatment with DETA-NO leads to phosphorylation and inactivation of the FOXO factor Foxo3a. To determine whether Foxo3a is in fact a transcriptional regulator of PGC-1α we have used a series of experimental approaches. Over-expression of a constitutive form of Foxo3a dramatically upregulated PGC-1α levels, whereas a siRNA directed against Foxo3a had the opposite effect. ChIP analysis showed that Foxo3a directly associates with the PGC-1α promoter and transient transfection assays indicated that Foxo3a activates the PGC-1α promoter through the same binding site that is required for NO activity. We therefore conclude that NO down-regulates PGC-1α expression through the PI3K/AKT/Foxo3a pathway.

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P-016

Bilirubin induces oxidative stress in neurons, which is prevented by nNOS inhibition

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High levels of unconjugated bilirubin (UCB) can be toxic to the central nervous system and oxidative stress is emerging as a relevant event in the mechanisms of UCB encephalopathy. We investigated if exposure of rat neurons to UCB leads to disruption of the redox status by a mechanism involving nNOS activation. Rat cortical neuronal cultures at 8-days *in vitro* were exposed to 50 or 100 μM UCB, for 4 h, at 37°C. To assess the role of NO, neurons were co-incubated with 100 μM UCB and 100 μM L-NAME, an inhibitor of nNOS. All the experiments were performed in the presence of 100 μM human serum albumin, used to solubilize UCB. Formation of protein carbonyls was assessed by slot blot analysis, reduced glutathione (GSH) by an enzymatic recycling assay and cell death was determined by quantification of LDH release using a commercial kit. NO production was evaluated by quantification of nitrite levels using Griess reagent, while the activity of nNOS was estimated by Western blot. Neuronal exposure to 50 or 100 μM UCB led to protein oxidation ($p < 0.05$) and decrease in GSH content ($p < 0.05$), resulting in enhanced cell death ($p < 0.01$). Oxidative disruption was accompanied by nNOS activation ($p < 0.05$), with consequent increase in NO production ($p < 0.01$). All UCB-induced effects were significantly counteracted by co-incubation with L-NAME. This study reinforces the involvement of oxidative stress in neuronal damage induced by UCB and demonstrates that cell lesion is mediated by nNOS activation, therefore, pointing to NO as a key element.

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P-017

Resveratrol prevents peroxynitrite-induced endothelial cells apoptosis by disrupting the mitochondrial pathway

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Resveratrol (3,4′,5-tryhidroxystilbene) is a phytochemical thought to account for red wine cardioprotective effects by exerting several biological activities. Atherosclerosis is a chronic inflammatory condition coupled with an increase on reactive species production, including peroxynitrite. In the present study, we aimed to investigate the biochemical pathway underlying both peroxynitrite-mediated endothelial cell death and resveratrol cytoprotective effects. Peroxynitrite (500 μM) triggers an apoptotic cell death that is prevented by resveratrol (1–50 μM) pre-treatment in a concentration-dependent manner, as indicated by nuclear morphology after Hoescht nuclei staining. Caspases activation analysis by cleavage of fluorogenic substrates shows that peroxynitrite cytotoxicity is associated with an activation of caspases-8, -9 and -3, suggesting that both mitochondrial and death receptors apoptotic pathways are involved. Resveratrol is able to efficiently prevent the peroxynitrite-induced caspases-9 and -3 activation, but such protective effect is much less noticeable in relation to caspase-8 activation. Additionally, we show that peroxynitrite increases Bax protein expression without affecting Bcl-2 protein expression, increasing therefore the ratio Bax/Bcl-2. This ratio decreased when cells were pre-incubated with resveratrol. Altogether, our results suggest that resveratrol may contribute to the proposed cardioprotective effects of red wine by disrupting the mitochondrial pathway involved in peroxynitrite-induced cell death.

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P-018

Antioxidative defense alterations in skeletal muscle during prolonged acclimation to cold: Role of L-arginine/NO producing pathway

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The effects of cold exposure on copper, zinc superoxide dismutase (CuZnSOD), manganese superoxide dismutase (MnSOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione S-transferase (GST), glutathione reductase (GR) and glutathione (GSH) as antioxidative defense (AD) components in skeletal muscle of rats were studied. A special attention has been paid to the influence of L-arginine/nitric oxide (NO) producing pathways on the modulation of AD in this tissue. For that purpose, adult Mill Hill hybrid hooded rat males were divided into two main groups: control, kept at room temperature ($22 \pm 1^{\circ}C$) and the group maintained at $4 \pm 1^{\circ}C$ for 45 days. Cold-acclimated group was divided into three sub-groups: untreated, L-arginine-treated (2.25% L-arginine·HCl in tap water) and N^{ω}-L-arginine-methyl ester-treated (0.01% L-NAME·HCl in tap water). The above AD parameters were determined in *musculus gastrocnemius* on days 1, 3, 7, 12, 21 and 45 of cold acclimation. The results demonstrated the upregulation of skeletal muscle AD by cold exposure. The most prominent cold-induced increase of MnSOD, CAT and GSH-Px activities observed in cold-exposed rats, was accelerated by L-arginine, while L-NAME delayed cold-induced changes. This undoubtedly indicates the effects of L-arginine/NO-producing pathway on the modulation of skeletal muscle AD and presumes that these effects were achieved through the stimulation of skeletal muscle oxidative metabolism.

P-019

HCV protein expression elicits deregulation of the calcium homeostasis between mitochondria and endoplasmic reticulum leading to intracellular redox unbalance

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Hepatitis C virus (HCV) infection induces a state of oxidative stress that is more pronounced than that in many other inflammatory diseases. In this study we used well-characterized cell lines inducibly expressing either the entire HCV open-reading frame or sub-genomic constructs to investigate the impact of viral protein expression on cell bioenergetics. It was shown that HCV protein expression has a profound effect on cell oxidative metabolism, with specific inhibition of complex I activity, depression of mitochondrial membrane potential and oxidative phosphorylation coupling efficiency, increased production of reactive oxygen and nitrogen species, as well as loss of the Pasteur effect. Importantly, all these effects were causally related to mitochondrial calcium overload, as pharmacological inhibition of mitochondrial calcium uptake completely reversed the observed bioenergetic alterations. The results presented along with a survey of the latest related literature allow one to conclude that the expression of HCV proteins causes deregulation of calcium homeostasis between the mitochondria-endoplasmic reticulum inter-organelle cross-talk. This event occurs upstream of further mitochondrial dysfunction, leading to alterations in the bioenergetic balance and nitro-oxidative stress. These observations provide new insights into the pathogenesis of hepatitis C and may offer new opportunities for therapeutic intervention.

P-020

Role of vascular endothelial growth factor and nerve growth factor in modulating the survival of skeletal muscle myoblasts exposed to oxidative stress

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The main objective of this study was to identify a specific role for VEGF and NGF towards the apoptotic cell death triggered by free radicals in skeletal muscle satellite cells, focusing on the involvement of the main growth factor receptors and of the anti-apoptotic proteins in this process. We demonstrated that the expression and release of high amount of VEGF by C2C12 myoblasts protects them from apoptosis induced by oxidative or chemical stress. This protection does not seem to be correlated to the modulation of the expression of VEGF receptors, but is clearly linked to the phosphorylation of KDR/Flk-1 receptor, the activation of NFkB and over-expression of small heat shock protein αB-crystallin. Regarding NGF, we demonstrated that this growth factor improves myogenin expression in the first phase of differentiative process of L6C5 rat skeletal myoblasts, and it determines fibre hypertrophy by increasing the fusion rate of skeletal myoblasts. The presence of exogenous NGF improves the overall cell survival after exposure to cytotoxic doses of H_2O_2, without modifying specifically the susceptibility to apoptosis. On the other hand, the utilization of specific TrkA and p75 inhibitors showed that the cross-talk between the cellular signalling activated by NGF receptors seems to be fundamental for myoblast survival.

P-021

Calorie restriction protects against age-related myocardial fibrosclerosis in the rat

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Currently, the free radical theory of ageing is one of the most popular. In parallel, many studies on the pathogenesis of fibrosis in different chronic pathological human diseases have demonstrated its association with an increased oxidative stress and often with ageing the fibrotic component of tissues increases. One of the few interventions effective in slowing down ageing is calorie restriction (CR) and the protection against the age-associated increase of oxidative stress remains one of the most outstanding hypotheses for explaining this action. We then studied CR effects on oxidative stress and fibrosis in the heart during ageing. We used male rats of different ages; CR was obtained by feeding animals at alternate days or with a 40% calorie reduced diet. In the heart of elderly rats with respect to young ones we found a significant increase of oxidative stress (4-hydroxynonenal content), parallelled by an increased fibrosis (transforming growth factor beta1 and collagen levels). In parallel, a significant increase of inflammation was evident

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with advancing age (IL-1 and TNF-alpha levels). CR protected from all these phenomena. As regards the signalling pathways involved, JNK and p38 did not show any variation with age, while ERK1 and 2 significantly increased. This age-related increase was also protected by CR. These data further support the hypothesis that CR protects against age-related fibrosclerosis at least in part by reducing oxidative damage and inflammation. The protection exerted by CR on oxidative stress and fibrosis can thus be considered in the prevention of age-related diseases with sclerotic evolution.

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P-022

Abrogation of *Leishmania infantum* mitochondrial peroxiredoxin impairs parasite infectivity

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Leishmania infantum is a protozoan parasite with relevance as human pathogen. *L. infantum* possesses one mitochondrial peroxiredoxin, *Li*mTXNPx, an enzyme previously reported to function as a peroxidase. Parasites depleted of this enzyme, *LimTXNPx*$^{-/-}$, were produced by DNA recombination. These mutants are morphologically similar to, display the same growth rate and have identical susceptibility to exogenous sources of peroxides (H_2O_2, *t*-bOOH, SIN-1) as wild type parasites. Interestingly, however, when performing *in vivo* infection assays, we observed that depletion of the mitochondrial peroxiredoxin impairs parasite infectivity by week 2 and 4 post-infection. Furthermore, by week 8, Balb/c mice infected with *LimTXNPx*$^{-/-}$ have almost undetectable levels of parasites in both their livers and spleens, in contrast to the high parasitemia levels of mice infected with wild type promastigotes. Looking for a rational for *LimTXNPx*$^{-/-}$ impaired infectivity, we are investigating whether *Li*mTXNPx might be implicated in *L. infantum* regulation of programmed cell death (PCD). This hypothesis was postulated with basis on previous reports stating that (i) mitochondrial peroxiredoxins of higher eukaryotes and possibly also of *Leishmania donovani*, regulate PCD and that (ii) PCD of *Leishmania* is an important mechanism for parasite establishment in the mammalian host. Understanding how *Li*mTXNPx affects parasite survival in the vertebrate host may provide us some answers about the mechanisms *L. infantum* makes use of to survive intracellularly.

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P-023

Quercetin enhances UVA-induced DNA damage in a rat fibroblast cell line

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Ultraviolet A (UVA) radiation from sunlight induces the production of reactive oxygen species (ROS), affecting a variety of cellular targets including the DNA. Quercetin, a flavonol present in many fruits, vegetables and beverages has been reported as a powerful antioxidant with an important role in prevention of carcinogenesis. The use of this compound, in topical formulations, could be of benefit in the prevention of skin damage produced by sunlight exposure. We investigated the effect of quercetin on DNA damage induced by UVA radiation in the rat subcutaneous fibroblast cell line, L929. Cells were irradiated by UVA light for 1 h, in the presence of quercetin and DNA damage assessed, in individual cells, by the alkaline single cell gel electrophoresis assay (Comet Assay). Our data showed that the combination of UVA with quercetin, at the three concentrations tested (20, 30 and 50 μM) enhances the level of DNA damage in a concentration-dependent manner. However, this effect seems not to be the same when cells are pre-incubated with quercetin, followed by irradiation, in the absence of the compound. We are investigating the mechanisms behind the observed harmful effect of quercetin together with UVA irradiation and trying to relate it, with the ROS levels, in both experimental conditions. The effect obtained suggests that, despite the well known antioxidant beneficial effects of quercetin in many different situations of oxidative stress, precautions should be taken if we think in the development of topical preparations with this compound, to be used on body areas exposed to sunlight.

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P-024

Role of lipid peroxidation in the toxicity of benomyl and carbendazim in liver of rats following acute exposure

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The benzimidazole fungucides, benomyl and its metabolite carbendazim, are used in controlling the pests of a variety of crops. The study was carried out to understand the role of benomyl and carbendazim in inducing oxidative stress leading to peroxidation of lipids and alterations in antioxidant system. Benomyl, carbendazim and benomyl + carbendazim were administered orally at doses of 1 g/kg, 0.64 g/kg and 0.1 g + 0.064 g/kg, for 1 day, respectively. Levels of malondialdehyde (MDA), an end product of lipid peroxidation (LPO), levels of reduced glutathione (GSH), the activities of antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), glutathione reductase (GSH-Rd), γ-glutamyl transpeptidase (GGT) and glutathione-S-transferase (GST) were determined in the homogenates of liver tissues. The results showed that the treatment with benomyl and carbendazim increased MDA levels, decreased GSH levels and SOD, CAT, GSH-Px, GGT, GST activities in livers as compared to control groups. According to the results we conclude that benomyl and carbendazim can cause oxidative stress in liver of rats. The experiments reported complied with the current laws and regulations of the Turkish Republic on the care and handling of experimental animals.

P-025

Dietary enrichment with almonds reduces oxidative stress and improves vascular function

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Epidemiological evidence suggests that diets rich in fruits, vegetables and pulses reduce the risk of cardiovascular disease. The Physicians Health Study demonstrated reduction of coronary heart disease mortality with regular nut consumption [1]. Almonds have low saturated fatty acid and high monosaturated fatty acid content and an absence of cholesterol. In addition, they are enriched in vitamin E, polyphenols and arginine, a precusor of the vasodilatory molecule, nitric oxide. Therefore, we have undertaken an almond enrichment study (25 g/d for 4 weeks followed by 50 g/d for 4 weeks) to evaluate possible mechanisms of cardiovascular benefits of almonds. Sixty non-smoking subjects were recruited into four groups; healthy male volunteers between the ages of 18–35 years, men at risk of cardiovascular disease between the ages of 18–35 years, mature men and women (> 50 years of age) and a control group without dietary almond enrichment. No significant effects of almond consumption were observed on total cholesterol, low density lipoprotein cholesterol (LDL-C) or high density lipoprotein cholesterol (HDL-C). In contrast, a 10% decrease in levels of the protein oxidation marker (carbonyls) was observed after 4 and 8 weeks in the mature subject group. A significant decrease in blood pressure was observed following almond consumption in all supplemented groups ($p < 0.05$). Mature subjects and those 'at risk' of CVD showed a significant increase in vascular flow-mediated dilation after 8 weeks of almond consumption which may be attributed to increased availability of nitric oxide.

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P-026

Evaluation of SOD and GPx activities in schizophrenia

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In recent years there is great evidence that oxygen free radicals play an important role in the pathophysiology of many neuropsychiatric disorders. In schizophrenia, antioxidant status could be altered as a consequence of both the evolution of the disease and the neuroleptic treatment. In the present study we investigated the activities of erythrocyte superoxide dismutase (SOD) and whole blood glutathione peroxidase (GPx) in 70 schizophrenic patients and 43 healthy volunteers, age and sex matched. We have observed significantly higher values of SOD activity (1486.45 ± 262.59 U/g Hb vs 1393.92 ± 250.73 U/gHb, $p < 0.05$) but normal values of GPx activity (38.86 ± 9.59 U/g Hb vs 44.24 ± 11.53 U/g Hb, $p > 0.05$) in the schizophrenia group compared with the control group. These results suggest an adaptative response to the increased superoxide radicals production.

P-027

Adaptation to oxidative stress in the chronic effects of cocaine and amphetamine

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Repeated abuse of the stimulant drugs cocaine and amphetamine is associated with extraneuronal dopamine accumulation in specific brain areas. Dopamine oxidative metabolism generates reactive oxygen species, namely H_2O_2. In this work we studied the involvement of oxidative stress in the chronic effects of cocaine and amphetamine in PC12 cells (a dopaminergic neuronal model), as compared to chronic H_2O_2 exposure. Long-term cocaine treatment largely, but not completely, protected the cells against a H_2O_2 challenge, whilst a decrement in intracellular ATP was observed. Complete H_2O_2 resistance of cells chronically exposed to H_2O_2 appears to involve changes in the activity of glutathione peroxidase (GPx), glutathione reductase (GRed) and superoxide dismutase (SOD), whereas chronic cocaine increased GPx activity only, possibly explaining the incomplete resistance to acute H_2O_2. PC12 cells chronically exposed to amphetamine initially exhibited changes in GPx, GRed and SOD activities that returned to control levels after 4 weeks of exposure. This biphasic effect may be explained by dopamine depletion evoked by amphetamine, and may explain the lower level of resistance to acute H_2O_2 of cells chronically exposed to amphetamine, in comparison with cells chronically exposed to cocaine. ATP/ADP levels decreased upon 2 weeks of exposure to the drugs and H_2O_2 and returned to control levels upon 3 weeks of exposure. Together, these results indicate that cellular adaptations of PC12 cells to cocaine and amphetamine are associated with changes in the activity of antioxidant enzymes, suggesting the involvement of oxidative stress in the chronic effects of these drugs of abuse.

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P-028

Identification of free radical oxidation products of 1-palmitoyl-2-lineloyl-phosphatidylethanolamine by LC MS/MS

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Lipid peroxidation—which encompasses oxidative degradation of phospholipids—occurs in cell membranes, resulting in alterations of the membrane's properties, such as fluidity and permeability. This process has attracted much attention due to the increasing evidence of its involvement in the pathogenesis of numerous illnesses, such as diabetes and cancer as well as age-related diseases, such as Parkinson's and Alzheimer's. In order to mimic the *in vivo* oxidation of membrane phospholipids, the Fenton reaction was carried out in 1-palmitoyl-2-lineloyl-phosphatidylethanolamine (PLPE) vesicles. The oxidation products formed were separated by reverse phase liquid chromatography coupled to a mass spectrometer using an acetonitrile gradient and characterized by tandem mass spectrometry (LC-MS/MS). The peroxidation products identified included species resulting from the insertion of oxygen atoms in the *sn-2* chain (long chain) and were, predominately, keto, hydroperoxide, hydroxy and poly-hydroxy derivatives, with high relative abundance of the keto derivatives. Products resulting from the shortening of the *sn-2* chain due to the cleavage of oxygen-centred radicals (short-chain) were also identified and comprised, mostly, aldehydes, hydroxy-aldehydes and dicarboxylic acids. Moreover, it was observed that, in PLPE, certain positions in the unsaturated chain are more susceptible to undergo radical oxidation, namely C-9 and C-12.

P-029

Glutathione reductase can be associated with high blood pressure in obesity

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The erythrocyte glutathione reductase (GR) is a cytoplasm enzyme which leads to the glutathione recycling protecting cells from oxidative stress. The objective was to study the relationship of erythrocyte GR activity with hypertension (HBP) of obesity (OB). A sample of 261 women from 25–79 years old ($M \pm SD = 49.37 \pm 12.76$), 85 with systolic HBP and 21 with diastolic HBP, 96 being obese (BMI > 30) were studied. The GR activity (μmol/min/grHb) was determined by spectrophotometer and BMI (Kg/m^2) and blood pressure by standardized methods. Statistical evaluation as done by Student *t*-test. The GR activity was lower in HBP when compared with normotensive (NT) (systolic HTA = 46.05 ± 18.18 and NT = 58.12 ± 20.03), $p < 0.001$. Although greater in OB (59.21 ± 20.09) vs non-obese (NOB) (51.86 ± 20.44), $p = 0.006$, GR activity was lower in HBP OB (OB 54.31 ± 22.91 vs NOB 44.32 ± 14.37, $p = 0.01$) than in NT OB (OB 63.49 ± 16.02 vs NOB 54.84 ± 21.16, $p = 0.006$). In conclusion, the GR activity could be implied in hypertension, due to failure of its oxidative stress defensive capacity in obese and non-obese women.

P-030

Erythrocyte glutathion reductase is a possible marker of cardiovascular age-related risk

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The erythrocyte glutathione reductase (GR) is an enzyme which leads to the glutathione recycling, protecting cell from oxidative stress being a marker of body riboflavin status. Pulse pressure reflects vasculature healthiness. The objective was to study the correlation of GR with age and cholesterol levels and its activity in hypertensive (HBP) and normotensive (NT) patients. A sample of 261 women from 25–79 years old ($M \pm SD = 49.37 \pm 12.76$), 85 with systolic HBP and 21 with diastolic HBP, was studied. The GR activity (μmol/min/grHb) was determined by spectrophotometry; blood pressure and serum lipids (International System U), by standardized methods. Statistical evaluation was done by Student *t*-test, ANOVA and Pearson correlation. GR was lower in HBP when compared with NT (49.09 ± 19.47 vs 57.47 ± 20.09, respectively) ($p = 0.002$), being different according to HBP JNC 7 class (NT = 58.54 ± 18.98; pre-HBP = 58.26 ± 20.81; HBP grade 1 = 46.82 ± 18.25; HBP grade 2 = 41.41 ± 16.89) ($p < 0.001$). GR was inversely correlated with age ($r = -0.782$, $p < 0.001$), pulse pressure ($r = -0.486$, $p < 0.001$) also with cholesterol total and LDL-c both in NT ($r = -0.305$, $p < 001$; $r = -0.311$, $p < 0.001$) and HBP ($r = -0.443$, $p < 0.001$; $r = -0.318$, $p = 0.002$). In conclusion, GR low activity can contribute to cardiovascular risk, being lower with ageing leading to vascular lesions supported by the association of GR and hypertension grade, pulse pressure and plasma cholesterol parameters. These results may support the oxidative/inflammatory involvement associated to HBP, as well as a possible deficit of riboflavin with age, justifying its inclusion as a food supplement.

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P-031

A new role for diphenyl diselenide: Protection against peroxynitrite-mediated endothelial cell death

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Superoxide and nitric oxide are generated by blood vessels and can rapidly react to produce peroxynitrite, a powerful oxidant that modifies lipoproteins making them more atherogenic. This study was designed to determine the effect of diphenyl diselenide, a new synthetic selenoorganic compound under investigation, on peroxynitrite-mediated endothelial damage, and compare it with the well known ebselen. Bovine aortic endothelial cells (BAEC) in primary cultures were treated with authentic peroxynitrite and cell viability, intracellular gluthatione content and gluthatione peroxidase activity were assessed. Experimental results showed that a long pre-incubation (24 h) with diphenyl diselenide (0.5 and 1 μM) protected endothelial cells from the damage promoted by peroxynitrite exposure, in a more effective way than ebselen. The intracellular levels of GSH were almost completely consumed by peroxynitrite and, although the compounds did not restore the normal levels, diphenyl diselenide *per se* increases significantly GSH in a concentration-dependent manner. This effect may be related with the significant increase in cellular GSH-Px activity promoted by this compound, which revealed to be even more active than ebselen. In conclusion, our data suggest a new role for diphenyl diselenide as a potential anti-atherogenic agent.

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P-032

Standardized methods for the measurement of biomarkers of oxidative stress, an effective tool for the modulation of individualized treatments in skin ageing and aesthetic medicine

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We here report the results of our studies, demonstrating the occurrence of a severe topical and systemic oxidative stress, induced by different mechanisms, during procedures of skin rejuvenation (physical, chemical, surgery) as reproduced on the animal model. *In vivo*, on patients undergoing dermo-aesthetic interventions (peeling, blepharoplasty, liposuction) we confirmed that, depending on dosage, duration of the application and individual *status*, treatments can either induce or inhibit cutaneous and systemic free radical (FR) processes. These data appear paradoxical, considering that skin ageing itself is characterized by increased formation of oxidation products, altered expression of antioxidant (AO) enzymes, decreased AO levels, enhanced transcriptional factors and stress proteins. Nevertheless, a progressive decline of the immune function and of the capability to produce physiologically active FR by competent cells, with consequent increased skin proneness to infections and inflammation, is also observed. The modulation of dermo-aesthetic procedures on an individual base, to decrease oxidative damage and induce a satisfactory stimulation of skin physiological production of FR, may therefore represent an ideal approach to the treatment of the aged skin. Combined oral/topical treatments with natural products with skin trophism (coenzyme Q_{10}, RRR-alpha-tocopherol, soy phospholipids, L-methionine, polyphenols), including AO, inhibiting excess oxidative damage, and mild pro-oxidants, accelerating wound healing and re-epithelization, and potentiating antimicrobial defence, represent an efficient tool to modulate FR production during ageing and diverse conditions damaging the skin, as demonstrated also on experimental models. To be effective though, treatments must be tailored on the specific physiological *status* of each patient and on the individual skin and systemic FR/AO status, to be thoroughly monitored during treatment and follow-up. Direct and indirect measurements of the main physiologically relevant FR and of biomarkers of oxidation can be performed by simple methods, by chemiluminescence, fluorescence, immunochemistry, spectrophotometry and HPLC techniques, on white blood/cutaneous cells, providing a new powerful tool for the dermatologist. These kind of preventive measurements, thoroughly standardized by Quality Control Assessment, are currently performed on a routine basis in our Institute, for patients admitted for dermocosmetology and tricology treatments, for plastic surgery and for autologous epidermal transplant in vitiligo and other pigmentary disorders.

P-033

***In vitro* strategies for predicting human acute toxicity: Screening oxidative cytotoxity by flow cytometry and high content assays**

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Sensitive and reliable methods to detect ROS or their effects are essential to understanding the role of oxidative stress in cytotoxicity. We present here a Cytomic Assay Panel for Oxidative Stress Screening that allows the multiparametric detection of relevant endpoints of oxidative activity and damage within the cells. Moreover, the 96-well format of these assays has the advantage of enhancing data generation while lowering the number of cells per experiment. We used a plate-reader interfaced flow cytometer (Cytomics FC500 MPL, Beckmann Coulter) and an automated epifluoresence microscope (In Cell Analyser 1000, GE Healthcare). The cytotoxicity of 21 toxic compounds was tested in three human cells cell lines, HepG2, SH-SY5Y and A.704, using dihydrodichlorofluorescein for the detection of intracellular peroxidative activity, Mitosox for the detection of intracellular levels of superoxide anion and the antibody-based OxyDNA Assay kit (Calbiochem) for the detection of the oxidized DNA base 8-oxoguanine. The results obtained in these *in vitro* endpoints were compared with previously reported data of human acute *in vivo* toxicity of the tested compounds, obtained from clinical, toxicological and forensic registries. Our assays were shown to have good statistical correlations with other standard *in vitro* and *in vivo* parameters when expressed either as IC50 (the molar concentration of a toxicant producing 50% of cell death) or EC50 (the molar concentration of a toxicant producing 50% of the maximal effect), which supports the value of the assays proposed to assess the oxidative cytotoxicity of toxicants. Therefore, we propose this Cytomic Assay Panel for Oxidative Stress Screening as a convenient *in vitro* tool for predicting human acute toxicity *in vivo*.

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P-034

Paraquat-induced oxidative stress and inflammation in the rat lung is effectively treated with sodium salicylate, leading to full survival of intoxicated animals

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Paraquat (PQ) toxicity involves the production of ROS and RNS, inflammation, disseminated intravascular coagulation and activation of transcriptional regulatory mechanisms. Considering that an antidote against PQ poisonings should counteract all these effects, sodium salicylate (NaSAL) may constitute an adequate therapeutic drug. To test this hypothesis, NaSAL (200 mg/kg ip) was administered to rats, 2 h after exposure to a potential lethal dose of PQ (25 mg/kg, ip). NaSAL treatment provided an effective inhibition of PQ-induced deleterious effects, namely oxidative stress, activation of transcription factors (NF-κB, p53, AP-1), platelet aggregation, cytochrome c release from mitochondria and consequent regulation of caspases activities. Importantly, this treatment was associated with a full survival of the PQ treated rats (extended for more than 30 days) in opposition to 100% of mortality by day 6 in PQ-only exposed animals. Thus, NaSAL seems to

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be an effective antidote for PQ poisonings. Of note, the administration of NaSAL was given 2 h after intoxication of rats with PQ, a lag time that confers realism for its application in humans.

P-035

Supplementation of vitamin E to the general public may do more harm than good

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Our recent meta-analyses taught us that (i) The ill-defined term 'oxidative stress' (OS) can not be defined by a universal term because commonly used criteria do not correlate with each other, and (ii) in comparison to matched 'controls', patients of different OS-related diseases (CVD, AD, DM) are associated with OS only when the level of OS is estimated by part of the studied criteria. Notably, CVD patients differed from 'control groups' only in terms of lipid peroxidation-related criteria and even these differences are quite small and hardly significant. Armed with this knowledge and with the concepts of decision analysis, we used a Markov model to test the question of whether or not to recommend vitamin E supplementation to the general public. Unlike meta-analysis, the use of a model enables adjustment of the study with respect to heterogeneities in both population and treatment (using registries of the general population). Using this approach, we conducted Monte-Carlo simulations of transformations between pre-defined health states. The results of these simulations indicate that indiscriminate supplementation of vitamin E to the general public may be harmful not only with respect to cardiovascular mortality (as previously suggested by both Miller et al. and Bjelakovic et al.) but also with respect to the 'quality-adjusted life years' (QALY). Preliminary analysis indicates that selective supplementation to people with low vitamin E levels may be beneficial. However, vitamin E supplementation to CVD patients (who are commonly believed to be under oxidative stress) is more harmful than supplementation to the general public.

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P-036

Antioxidant protection in autism

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Autism is a severe neurodevelopmental disorder defined by social and communication impairments and stereotyped behaviours. The underlying aetiology of autism is unknown. It is suggested that autism may result from an interaction between genetic, environmental and immunological factors, with oxidative stress as a mechanism linking these risk factors. Knowing that the brain is highly vulnerable to oxidative stress due to its limited antioxidant capacity and higher amounts of lipids and iron, we investigated the activity of the erythrocyte superoxide dismutase (SOD) and whole blood glutathione peroxidase (GPx) in patients with typical and atypical autism. We investigated 41 children, 21 with typical autism (age 4.82 ± 0.33 years) and 20 with atypical autism (age $8{,}99 \pm 0.81$ years) followed at the Paediatric Psychiatry Clinics in Cluj-Napoca. The control children were free of any neuropsychiatric disorder, age and sex matched. We observed normal level of GPx activity in both typical (44.66 ± 2.92 U/g Hb) and atypical (42.02 ± 2.34 U/g Hb) autistics in comparison with age matched controls (47.63 ± 3.30 U/g Hb and 44.23 ± 2.74 U/g Hb, respectively). On the other hand, the activity of SOD was significantly decreased only in children with atypical autism compared with matched age controls (1154.28 ± 79.62 U/g Hb vs 1395.10 ± 60.18 U/g Hb, $p < 0.05$). In conclusion, our results indicate that the antioxidant defense is altered in atypical autism, but not in typical autism.

P-037

Insulin neuroprotection against oxidative stress involves Akt/GSK-3β signalling pathways and changes in protein expression

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We recently demonstrated that insulin stimulates antioxidant defenses and restores energy metabolism in cortical neurons, thus protecting against oxidative stress, a deleterious condition associated with diabetes and neurodegenerative diseases. In this study, we analysed the effect of insulin on neuronal insulin- and insulin growth factor-1 receptor (IR and IGF-1R, respectively)-mediated intracellular signalling pathways upon ascorbate/Fe^{2+}-induced oxidative stress in rat primary cortical neurons. We showed that both IR and IGF1R are expressed in these neurons. Moreover, insulin prevented oxidative stress-induced decrease in IR and IGF-1R Tyr phosphorylation, although it did not affect IR and IGF-1R phosphorylation *per se*. Despite no significant changes in extracellular signal-regulated kinase (ERK) signalling upon ascorbate/Fe^{2+} treatment, insulin also prevented oxidative stress-induced inactivation of Akt and decreased GSK-3β Tyr216 (glycogen synthase kinase-3β, active form), which has been reported to stimulate protein synthesis and decrease apoptosis. Thus, we analysed the changes in mRNA and protein expression levels of 'candidate' proteins involved in antioxidant defense, glucose metabolism and apoptosis. Under oxidative stress, insulin prevented the increase in glutathione peroxidase-1 and the decrease in hexokinase-II expression, supporting our previous findings of increased antioxidant activity of glutathione redox cycling and glycolysis stimulation. Insulin also precluded a decrease in expression of Bcl-2, an increase in caspase-3 expression and activity and in DNA fragmentation. These data suggest that insulin-mediated activation of IR and IGF-1R stimulates PI-3K/Akt and inhibits GSK-3β signalling pathways, regulating neuronal antioxidant defense, glucose metabolism and anti- or pro-apoptotic proteins, thus protecting against deleterious effects induced by oxidative stress in neurons.

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P-038

Oxidative stress in type 2 diabetic GK rat retina during ageing

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Age-related increase in oxidative stress and subsequent neurodegeneration appear to be involved in the pathophysiology of long-term diabetic complications affecting the retina. Diabetic retinopathy is one of the causes of blindness in ageing in the western countries. In this study we investigated the effect of ageing on the vulnerability of the retina to oxidative damage in Goto-Kakizaki (GK, a model of type 2 diabetes) vs normal Wistar rats, at 3 (young), 6 (adult) and 20 months (age). We observed that blood glucose levels increased with ageing in type 2 diabetic GK rats and that the increase in lipid and protein oxidation in young diabetic rat retina was primarily correlated with a decrease in vitamin E antioxidant defence. At this age, GK rat retina showed an increased activity of glutathione-S-transferase (GST), suggesting a response to increased oxidation. Interestingly, adult GK rat retinas were less affected by lipid and protein oxidation, probably due to increased vitamin E and GSH regeneration. At 6 months of age, GK rat retina may have developed protective mechanisms against hyperglycemia-induced oxidative damage. Conversely, retinas from aged GK rats were more prone to oxidative injury resulting from a reduction of vitamin E and glutathione redox cycle. These results suggest that oxidative stress

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is an early event in the pathogenesis of diabetic retinopathy. Moreover, age-dependent decrease in endogenous antioxidants may underlie retina oxidative degeneration in type 2 diabetic retinopathy.

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P-039

Postmenopausal syndrome and its influenting by polyphenolic extract, Pycnogenol

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Post-menopausal symptoms (PMS) include hot flashes, fatigue, night sweats, mood changes, insomnia, myalgias, arthralgias, anxiety and sexual dysfunction. Sixty-two women with PMS were supplemented with 100 mg per day Pycnogenol® or placebo (PL) over a period of 3 months in a randomized, placebo-controlled, double-blind study. Pycnogenol® (PYC), French maritime pine bark extract, containing numerous polyphenols, stimulating production of endothelial nitric oxide synthase in cells. We examine the effect of PYC on PMS symptoms. Sixty women were supplemented with PYC in dose 100 mg per day or placebo over a period of 3 months in a randomized, placebo-controlled, double-blind study. Patients were examined by standard questionnaire Menopausal Rating Scale at the start of trial, 3 months after treatment and 1 month after the end of treatment period. The effect of PYC administration as whole effect on all 11 evaluated PMS symptoms as well as on grouped somatic, psychic and urogenital problems was determined as significant by Friedman test in comparison to the run-in period (1–4 weeks). PL administration did not cause as a whole a significant effect. The significance between the effect of PYC and PL was observed for all symptoms in weeks 12, 13 and 16. Our results allow us to conclude that PYC offers women a natural supplement for relieving post-menopausal symptoms.

P-040

UPF peptides, antioxidative glutathione analogues: Design, free radical scavenging *in vitro* and toxicity to K562 cells

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Glutathione (GSH, γ-Glu-Cys-Gly) is the major intracellular low molecular weight antioxidant. Decreasing of GSH pool has been shown to be a part of many pathological events but the exogenous compensatory administration of GSH is problematic. The purpose of this work was to create more stable and effective antioxidants than GSH and investigate their structure-activity relations. By adding a fourth amino acid to the GSH molecule, we designed and synthesized 16 tetrapeptidic glutathione analogues called UPF peptides. UPF peptides were far more active hydroxyl radical scavengers than glutathione (EC_{50} of GSH: 1231.0 mM; EC_{50} of UPF peptides: from 0.03–35 μM) and showed improved anti-radical efficiency towards DPPH radical. The most effective structural modification was the substitution of the γ-Glu with the α-Glu moiety in GSH backbone resulting in EC_{50} values of sub-micromolar range, measured by terephthalic acid method. UPF peptides did not affect the viability and membrane integrity of human erythroleucemia K562 cells. In conclusion, UPF peptides may have potential as protective molecules against lesions caused by excessive oxidative stress.

P-041

Inefficient glutamate cysteine ligase up-regulation: A novel mechanism to differentiate necrotizing from oedematous pancreatitis

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Glutathione depletion is a key factor in the development of acute pancreatitis. Our aim was to study the regulation of glutamate cysteine ligase, the rate-limiting enzyme in glutathione synthesis, in oedematous or necrotizing pancreatitis in rats. Glutathione levels were kept low in necrotizing pancreatitis for several hours, with no increase in protein and mRNA levels of glutamate cysteine ligase sub-units, despite binding of RNA polymerase II to their promoters and coding regions. Furthermore, the survival signal pathway mediated by ERK and c-MYC was activated and c-MYC was recruited to the promoters. The failure in gene up-regulation seems to be due to a marked increase in cytosolic ribonuclease activity. In contrast, in oedematous pancreatitis glutathione levels were depleted and rapidly restored and protein and mRNA expression of glutamate cysteine ligase increased markedly due to enhanced transcription mediated by recruitment of c-MYC, NF-κB and SP-1 to the promoters. No increase in cytosolic ribonuclease activity was found in this case. We propose a novel pathophysiological mechanism to differentiate necrotizing from oedematous pancreatitis, which is the inefficient up-regulation of glutamate cysteine ligase caused by increased cytosolic ribonuclease activity in the severe form of the disease. This mechanism abrogates a rapid recovery of glutathione levels.

P-042

High extracellular glucose levels modulate the uptake of organic cations in Caco-2 cells

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Chronic exposure to high extracellular glucose concentration has been suggested to alter regulation and functional activity of proteins through oxidative stress pathways. According to results from a previous work, oxidation-reduction pathways were thought to be involved in intestinal organic cation uptake modulation. The present work was performed in order to evaluate the influence of different extracellular glucose concentrations on the intestinal organic cation absorption. For this purpose, the effect of 5.5 mM glucose and 25 mM glucose (HG) in culture media on 1-methyl-4-phenylpyridinium (MPP^+) uptake was evaluated in Caco-2 cells. Expression of hOCT1 and hEMT was investigated in both types of cells. Modulation by compounds with different redox potential (procyanidins and glutathione) was also assessed. ^{3}H-MPP^+ uptake as well as its affinity for the transporter were significantly decreased in HG cells. In HG cells, only hEMT transcription was affected (reduced). Redox changing interventions affected MPP^+ uptake, both in control and in HG cells. The results reinforce our previous conclusion, showing that modifications in the cellular oxidative state modulate MPP^+ uptake by Caco-2 cells. Such modifications may reflect in changes of nutrient and drug bioavailability.

P-043

Pyridine-containing macrocyclic copper(II) complexes: Studies on their potential role as superoxide scavengers

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The involvement of superoxide anion in several pathologies justifies the development of low molecular-weight SOD mimetics for therapeutic purposes. Most of these antioxidants have a redox active metal centre [e.g. Cu(II)] enclosed in a stable ligand. Macrocyclic compounds have presented advantages both on the SOD-like activity and on biological stability. The presence of coordination sites belonging to nitrogen heteroaromatic rings, like pyridines, seems to be important for a SOD-like activity not affected by biological chelators. The π-electrons in the pyridine ring may also favourably respond to the Cu(II)-$O_2^{\cdot -}$ interaction. In this work, four pyridine-containing macrocycles with a ring size of 14–16 atoms $MePy[14]eneN_4$ (L1) $ac_3Py[14]eneN_4$ (L2), $Py[15]eneN_5$ (L3) and $Py[16]eneN_5$ (L4) were synthesized following previous

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procedures and their copper(II) complexes were prepared. These ligands present high stability constants for copper(II), as determined by potentiometric methods. The superoxide scavenging activity of the complexes was studied using two different methods: the nitroblue tetrazolium reduction and the dihydroethidium oxidation. Cu(II)-L1 and Cu(II)-L3 have shown the ability to scavenge $O_2^{\cdot -}$, with IC_{50} values in the low micromolar range. Cu(II)-L3 presented the lowest IC_{50}. The cytotoxicity profiles of the complexes were evaluated in V79 Chinese hamster cells, using the MTT assay. The complexes were not considerably toxic up to 100 μM, with the exception of Cu(II)-L2. Among the complexes studied, Cu(II)-L3 presents a number of important characteristics. It has an effective superoxide scavenging activity, a high stability constant and a low cytotoxicity, appearing to be a promising superoxide scavenger.

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P-044

Decrease of GLUT1-mediated glucose uptake in endothelial cells in response to oxidative stress

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Increased oxidative stress is implicated in the pathogenesis of diabetic retinopathy. The goal of this study is to assess the regulation of glucose transport by oxidative stress. Retinal endothelial cells were subjected to oxidative stress by incubation with glucose oxidase. Protein carbonyl formation was used as an indicator of oxidized proteins. GLUT1 mRNA levels were determined by real-time RT-PCR. GLUT1 protein levels were detected following biotinylation of the membrane proteins. The glucose transport activity was measured by 3H-DOG uptake. Incubation of endothelial cells with glucose oxidase leads to an accumulation of oxidized proteins. Oxidative stress induces a decrease in the GLUT1 mRNA and protein levels. Significantly, glucose transport is decreased in oxidative stress. This result is in agreement with the decreased expression of the protein at the plasma membrane as well as with its decreased halflife. The inhibition of proteasome upon oxidative stress restores glucose transport to basal levels. In conclusion, the data suggest that sub-cellular redistribution of GLUT1 under conditions of oxidative stress contribute to disrupt glucose homeostasis in diabetes.

P-045

Determination of 3-N-tyrosine in human saliva by high-performance liquid chromatography (HPLC) with electrochemical detection.

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3-Nitrotyrosine (Ntyr) is considered as a biomarker of the generation of reactive nitrogen species (RNS). However, it is still difficult to determine its concentration in biological samples, in particular in saliva. Saliva is the first barrier against free radicals in the human organism and the determination of salivary Ntyr could tell us how saliva deals with nitric oxide-mediated damage. High performance liquid chromatography with electrochemical detection (HPLC-ECD) offers an attractive alternative to measurement of protein oxidation and nitration products. To develop a reliable and high-throughput method, we optimized the conditions for HPLC-ECD. The preparation of human saliva samples consisted of incubation with Fenton reagent, protein precipitation, enzymatic digestion and Ntyr determination by HPLC-ECD. The best separation of Ntyr was achieved using a highly acidic mobile phase (pH 3.1). Our protocol is suitable for analysing saliva samples to study RNS production.

P-046

Lipid peroxidation inhibition, free radical scavenging activity and electrochemical behaviour of a dihydroxylated di(hetero)arylamine

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The skin provides the first line of defence against oxidative damage induced by environmental factors, having an elaborated antioxidant system designed to deal with free radicals and oxidative stress. However, under severe stress conditions this biological response is not sufficient, leading to oxidative damage and, in consequence, to skin disorders, immunosuppresion, premature skin ageing and ultimately cancer. In these circumstances, antioxidants may play an essential role in enhancing the antioxidant system and thus preventing carcinogenesis. Considering treatment limitations and the high number of cancer patients, the development of new therapeutic strategies are urgently required. In this study, the antioxidant properties of ethyl 3-(2,4-dihidroxyphenylamino)benzo[*b*]thiophene-2-carboxylate, synthesized by us, were evaluated through their lipid peroxidation inhibition capacity, free radical scavenging activity and electrochemical behaviour. The chemical assays gave the following EC_{50} values: 211 μM for reducing power and 145 μM for radical scavenging activity of DPPH radicals (under the same conditions, the EC_{50} values for α-tocopherol were 158 and 92 μM). The biochemical assays used as models for the lipid peroxidation damage in biomembranes revealed the following EC_{50} values: 44 μM for inhibition of β-carotene bleaching in the presence of linoleic acid radicals (6 μM for α-tocopherol), 99 μM for inhibition of erythrocytes haemolysis mediated by peroxyl radicals (16 μm for α-tocopherol) and 63 μm for inhibition of thiobarbituric acid reactive substances (TBARS) formation in brain cells (11 μM for α-tocopherol). Cyclic voltammetry of the compound in acetonitrile/Pt electrode, at fast scan rates, showed an irreversible oxidation system with three anodic peaks at $E_{a1} = 0.82$ V, $E_{a2} = 1.59$ V and $E_{a3} = 1.77$ V. After the first scan a new oxidation/reduction system appears at lower potentials, $E_{a4} = 0.16$ V and $E_{c4} = 0.05$ V, that increases in intensity with the first five scans. At slow scan rates, below 0.1 V/s, this new system is not observed, pointing out a slow homogenous reaction after the first electron transfer.

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P-047

Electrochemical study of diarylamines in the benzo[*b*]thiophene series

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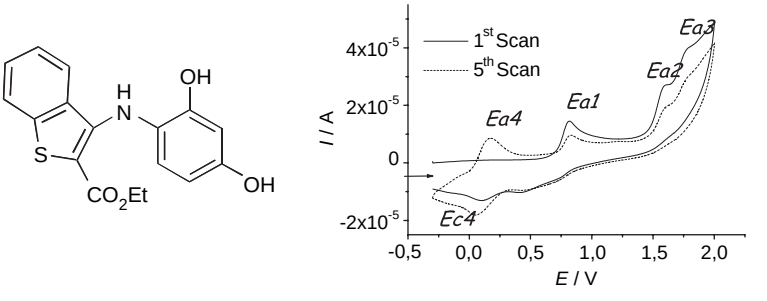

Figure 1.

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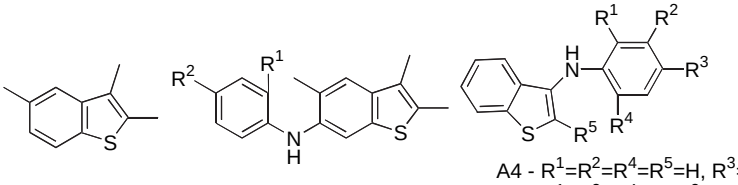

A1

A2 - R^1=F, R^2=H
A3 - R^1=H, R^2=OMe

A4 - R^1=R^2=R^4=R^5=H, R^3=F
A5 - R^1=R^2=R^4=H, R^3=F, R^5=CO_2Et
A6 - R^1=R^3=OMe, R^2=R^4=H, R^5=CO_2Et
A7 - R^1=R^5=H, R^2=R^3=OMe, R^4=Br

Figure 2.

The search for new molecules with antioxidant properties is a very active domain of research, since they can protect the human body from free radicals and retard the progress of many chronic diseases, such as vascular diseases, some forms of cancer and oxidative stress responsible for DNA, protein and membrane damage. Differently substituted diarylamine derivatives of benzo[*b*]thiophene were synthesized using C–N palladium-catalysed cross-couplings [1,2]. Recently, we have described the structure–activity relationship of some diarylamines in the benzo[*b*]thiophene series as antimicrobial [3] and antioxidant agents [4]. Here we extend this evaluation to the study of their electrochemical behaviour, regarding specially the influence of the compound structure, such as the position of the arylamination, the presence of different groups on the phenyl ring (Br, F, OMe) and on the thiophene ring (H, CO_2Et). The electrochemical studies of the above compounds were achieved in acetonitrile/TBAP, with a platinum electrode. At low scan rate, the cyclic voltamogram of benzo[*b*]thiophene *A1* presents two typical irreversible oxidation processes at $E_{a1} = 1.49$ V, $E_{a2} = 1.84$ V, controlled by the diffusion of the substract. The same pattern is observed in most of the compounds studied, however at different potentials. For example, the arylamination on the electron rich thiophene ring decreases the oxidation potential (*A2* vs *A4*) and the introduction of an electron withdrawing group in the thiophene moiety, CO_2Et, increment 0.3 V in the first oxidation potential (*A4* vs *A5*). This changes will influence the reducing power, with the highest effect found for compounds with lower oxidation potentials [5].

Research Project POCI/QUI/59407/2004 (FCT-Portugal).

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P-048

The thioredoxin TRX-1 is involved in ASJ-dependent starvation stress responses in *C. elegans*

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The *C. elegans* gene *trx-1* encodes a thioredoxin that is expressed in the ASJ ciliated chemosensory neurons. We have previously shown that *trx-1* deletion mutants are short-lived, whereas animals expressing a translational *trx-1::gfp* fusion transgene are long-lived relative to wild type (WT). We report here that trx-1 participates in ASJ-dependent starvation stress responses. We find that *trx-1::gfp* expression in ASJ neurons is up-regulated in WT animals under conditions of starvation and in the developmentally arrested dauer larval stage, implicating *trx-1* in ASJ-dependent stress resistance mechanisms. Interestingly, in *daf-11* guanylyl cyclase mutants, *trx-1::gfp* expression in ASJ neurons is constitutively up-regulated, indicating that depletion of cGMP up-regulates *trx-1* expression in ASJ. Similarly, deletion of *trx-1* alters the dauer formation constitutive (Daf-c) phenotype of *daf-11* mutants, supporting the involvement of *trx-1* in ASJ-dependent starvation stress responses (e.g. dauer arrest). In addition, we observe that deletion of *trx-1* partially suppresses the Daf-c phenotype of *daf-28* insulin-like mutants, suggesting that TRX-1 affects the insulin/IGF-like signalling (IIS) pathway in ASJ neurons to promotes dauer arrest. Taken together, it seems likely that the up-regulation of *trx-1* under conditions of starvation forms part of a stress response elicited to protect *C. elegans* in harsh food-deprived environments. As a consequence, this ASJ-dependent stress response would promote longevity and dauer formation at the cost of reproduction and growth. Current efforts are directed towards studying the genetic interactions of *trx-1* with genes involved in ageing and dauer pathways to identify the upstream regulators of *trx-1* expression.

P-049

Measurement of total antioxidant status in biological fluids as indicator of the activity of the antioxidant system

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Oxidative damage has been implicated in the aetiology or pathogenesis of a large number of diseases, in tissue injury as well as in the ageing process. To reduce free radical damage, control mechanisms are present, the most important is the antioxidant system. Antioxidants are a heterogeneous group of substances such as enzymes, other proteins and a range of compounds such as vitamins, uric acid, glutathione, flavonoids, phenols and carotenoids. The antioxidant defenses interact to form an integrated system and it is of great interest to measure the total antioxidant status (TAS) as an indicator of the functioning of the entire system. We report here examples of the applicability of an assay kit to the quantitative determination of total antioxidant status in serum or plasma samples. Serum/plasma samples were collected, stored and assayed with a two-reagent colourimetric total antioxidant status assay kit. Measurements were taken at 600 nm. The linearity of the assay was 2.5 mmol/L. Measurement of TAS in geriatric ($n = 77$) and normal working ($n = 156$) populations data indicated significant reduction of serum total antioxidants in geriatrics when compared with the working age group (1.284 mmol/L vs 1.536 mmol/L, $p < 0.05$). In both groups the values were higher in males when compared with female subjects. Measurement of TAS in plasma of normal volunteers ($n = 16$) before and after 60 days of vitamin supplementation showed a statistically significant increase post-supplementation (from 1.56 mmol/L to 1.62 mmol/L). In conclusion, data show applicability of this total antioxidant status kit to the assessment of the integrated antioxidant system.

P-050

Oxidation of Tyr-Leu and Leu-Tyr: Identification of spin-trapped free radicals by tandem mass spectrometry

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Reactive oxygen species formed during oxidative stress are implicated in diseases and ageing. Being proteins, one of the oxidation targets resulting in their structural modifications and loss of function. $HO^{\bullet}$, the most reactive radical, when generated by different sources (radiolysis or metal-catalysed oxidation) leads to different intermediate free radicals and oxidation products. These free radicals have short-living time, making them difficult to detect. Spin traps, namely DMPO, form stable adducts, usually identified by EPR, with these radicals. Mass

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spectrometry has been used for the characterization of lipid spin traps adducts but less extensively for the proteins radical adducts. Information about these radicals is essential to understand protein *in vivo* oxidation. YL and LY (*m/z* 295) were oxidized under Fenton's conditions (Fe (II)/H_2O_2) and the free radicals detected as DMPO adducts were monitored by HPLC-MS and characterized by HPLC-MS/MS using a Linear Ion trap LXT (Thermo). HPLC-MS and HPLC-MS/MS allowed the identification of carbon and oxygen DMPO adducts of YL and LY radicals in its unmodified and oxidized forms (with one and two oxygen atoms for YL and with one oxygen atom for LY). Positional isomers were identified since carbon and oxygen centred radicals were located in different positions in the aa Y. Also radicals in the aa L were observed. However, the isomers with the radicals in the aa Y showed higher relative abundance. DMPO adducts with radicals that resulted from peptide backbone cleavage were also identified. This study attempts to elucidate which radicals were formed under Fenton's conditions.

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P-051

Ethanol and GSNO modulate NMDA-induced nitric oxide production in hippocampal slices

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Nitric oxide ($^{\bullet}NO$) is a small, lipophilic and highly diffusible neurotransmitter implicated in a number of neuronal events, including regulation of neurotransmitter release, neuronal plasticity and neuronal degeneration and survival. Increased activity of the glutamatergic NMDA receptor (NMDAr) is the dominant mechanism by which $^{\bullet}NO$ is generated in the brain and we have provided evidences for the concentration dynamics of $^{\bullet}NO$ following NMDAr stimulation in the hippocampus, a brain structure involved in learning and memory formation. We are now reporting changes in the rate and pattern of $^{\bullet}NO$ production in NMDA-stimulated hippocampal slices in the presence of candidate exogenous (ethanol) and endogenous (GSNO) modulators. Using an electrochemical approach, hippocampal slices were stimulated with NMDA in the presence of increasing concentrations of ethanol. Perfusion of ethanol caused a marked concentration-dependent decrease in $^{\bullet}NO$ production (from 0.1% to 1% v/v), a result also observed with increasing periods of slices exposure to the same drug (namely 10, 30 or 60 min). In another set of experiments preliminary results showed that the proposed NMDAr modulator GSNO reduced $^{\bullet}NO$ production following slice stimulation with NMDA after very brief incubation periods (10 min). Although further studies are required to clarify the pathological and physiological implications on the modulation afforded by these agents, our results clearly demonstrate that the known NMDA antagonist effect of ethanol (and GSNO) impacts on the modulation of $^{\bullet}NO$ concentration, and therefore interferes with local $^{\bullet}NO$ biological activity. These results also suggest a mechanism for the ethanol effects in hippocampus-dependent memory and learning processes.

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P-052

Cigarette smoking increases oxidative stress in prostate cancer patients: A preliminary study

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Cigarette smoke is an oxidant, creating free radicals that are involved in a diversity of important phenomena in the process of carcinogenesis. Prostate cancer, a leading cause of cancer-related deaths, has been associated with increased levels of oxidative stress during its development and progression, however a correlation between cigarette smoking and prostate carcinogenesis has not been established yet. This study was directed to evaluate the effect of cigarette smoking on oxidant/antioxidant status of patients with prostate cancer. The Total Antioxidant Status (TAS), glutathione, glutathione peroxidase activity, glutathione reductase activity, nitric oxide, vitamins E and A, uric acid, malondialdehyde and carbonyl groups were analysed in the plasma and/or in the erythrocytes of 14 prostate cancer patients, five smokers and nine non-smokers and in six controls, two smokers and four non-smokers. Our preliminary results show a significant decrease in TAS and glutathione and an increase in MDA in the prostate cancer smoker group, when compared with control. However, in the smoker control group we found an increase in TAS when compared with non-smokers. These results suggest that cigarette smoking may be involved in prostate cancer development, as we observed down-regulation of critical antioxidant defenses and increase in oxidative lesion. However, further research is necessary to better clarify the influence of cigarette smoking and oxidative stress in prostate carcinogenesis.

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P-053

Oxidative burst in neutrophils from hemochromatosis patients

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Classic hereditary haemochromatosis is an autosomal recessive disorder of iron metabolism that causes the progressive cellular accumulation of iron in cells. Because humans cannot increase the excretion of iron, the resulting iron overload may eventually damage tissues and organs, generally by the fourth to fifth decades of life. Neutrophils are the most abundant leukocytes in the blood and participate actively in the innate host defense response. Relatively little is known about the effect of hereditary haemochromatosis on the reactivity of neutrophils. When neutrophils are activated, they initiate a 'respiratory burst', resulting in the formation of ROS like superoxide radical ($O_2^{\bullet-}$) via action NADPH-oxidase, with subsequent production of hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), peroxyl radical ($ROO^{\bullet}$) and hypochlorous acid (HOCl) (via myeloperoxidase). Considering that these patients have an iron overload, this condition may have an increased importance in the production of $HO^{\bullet}$ through the Fenton reaction. The aim of the present study was to evaluate the sensitivity of isolated human neutrophils from haemochromatosis patients to the stimulation of oxidative burst by phorbol myristate acetate (PMA) as compared to control healthy humans. The measurement of neutrophil burst was undertaken *in vitro*, by chemiluminescence, by monitoring the oxidation of luminol by neutrophil-generated ROS and RNS in haemochromatosis patients and healthy individuals. The obtained results showed a higher sensitivity to PMA activation by neutrophils from hemochromatosis patients.

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P-054

Mitochondrial biogenesis during ageing

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Mitochondria serve a critical function in the maintenance of cellular energy supplies. Alterations in mitochondrial function are responsible for a range of inherited and acquired human diseases and are implicated in the ageing process. There's a correlation between energy demand

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and mitochondrial abundance, pointing to sophisticated regulatory mechanisms that control mitochondrial biogenesis. The mitochondrial biosynthetic programme appears to involve the integration of multiple transcriptional regulatory pathways, controlling expression of both nuclear and mitochondrial genes in a tissue and stimulus specific way. We used young and old male Wistar rats and we investigated the effect of oxidative stress on mitochondrial biogenesis. We've found a significantly decrease in the levels of peroxisome proliferator-activated receptor–coactivator1 (PGC-1), nuclear respiratory factor–1 (NRF-1) and cytochrome c (Cytc) and an increase in the levels of mitochondrial transcription factor A (Tfam) in the heart of the old rats. In the liver we found a significant decrease of the levels of Tfam and of NRF-1 and Cytc in the old rats compared to young ones, but this wasn't accompanied by a change in the levels of PGC-1. As pyruvate stimulates an increase in cytosolic Jun-N-terminal kinase (JNK) activity we evaluated the possible activation of it. We found a significant increase of JNK phosphorylation only in the liver in old rats compared to the young. In conclusion, increasing ROS production involves a decrease in the biogenesis of the mitochondria in the old animals.

P-055

Nitrite and red wine interact in the stomach yielding nitric oxide which induces local smooth muscle relaxation

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The stomach may be a source of new bioactive molecules with impact in physiologic and pathologic processes. Local conditions favour the reaction among dietary products. We show that ethyl nitrite forms from the reaction of red wine or distilled alcoholic drinks with nitrite in simulated gastric juice. The identification and quantification of ethyl nitrite was achieved by GC-MS. For ordinary-achievable concentrations of ethanol and nitrite in the stomach the concentration of ethyl nitrite can reach up to tens of μM. Indeed, *ex vivo* studies showed relaxation of rat stomach fundus strips and femoral artery rings when incubated with wine and nitrite mixtures. This effect was quantitatively assigned to the locally formed ethyl nitrite. A dose-response relaxation curve indicated that 50% of maximum relaxation was achieved with 250 and 100 nM of ethyl nitrite in stomach strips and artery rings, respectively. Mechanistically, the relaxation effect was assigned to the chemical (non-enzymatic) production of nitric oxide as supported by: (1) the demonstration of nitric oxide release from ethyl nitrite using selective electrodes; (2) the absence of relaxation in the presence of the soluble guanylyl cyclase inhibitor, ODQ; and (3) the inability of L-NAME, an inhibitor of nitric oxide synthase, to modulate the relaxation. In conclusion, these results suggest that ethanol (from alcoholic drinks) and nitrite can interact in the stomach leading to the production of ethyl nitrite, which, in turn, by releasing nitric oxide, induce local relaxation on the stomach muscle layer. If absorbed into the blood stream more widespread effects are expected. These findings reveal a new pathway for the biological effects of dietary nitrite encompassing its interaction with red wine with impact on human physiology.

P-056

Studies of dequalinium efect on redox balance in human leukemic cells

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Dequalinium (DQA) has been proposed as a selective anti-tumoural agent due to its preferential accumulation in mitochondria of tumour rather than of normal cells. Mitochondria, which regulate main cellular events, such as cell life or death, seems an adequate target for tumour cell eradication. To understand the anti-tumour mechanism of DQA, we are studying its effect on two human leukaemia cell lines: K562 (chronic myeloid leukaemia in blastic crisis), which is very resistant to apoptosis, and NB4 (acute promyelocytic leukaemia). DQA produces early cell alterations which are time- and DQA concentration-dependent, being more sensible in NB4 than in K562 cells. NB4 cells died by apoptosis, on the contrary, K562 cells only undergo necrosis when treated with high DQA concentrations. DQA causes mitochondrial dysfunction affecting the transmembrane potential as well as the ATP synthesis. DQA also affect the redox balance which results in oxidative stress by increasing ROS production and decreasing GSH content. This study suggests the DQA potential as a selective anti-leukaemic agent for acute promyelocytic leukaemia cells treatment.

P-057

Effect of alpha-lipoic acid and vitamin E in the oxidative stress associated with an acute process of hypoxia-reoxygenation

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The purpose of this study was to observe the effect of the treatment with alpha-lipoic acid (LA) and vitamin E in rats exposed to hypobaric hypoxia followed by reoxygenation. The Wistar rats (180–200 g) were divided as follows: a control group kept in normoxia and three groups of animals which were treated, for 7 days prior to exposure, with LA 50 mg/kg/day; vitamin E 50 mg/kg/day; 0.9% NaCl solution. The altitude was of 2500 m and was simulated in a baric chamber (24 h). To achieve reoxygenation, the animals were brought back under normoxia conditions for 2 h. Subsequently, venous blood was collected and the following oxidative stress markers were determined by spectophometric analysis: malondialdehyde, carbonyl proteins, hydrogen donor capacity and the superoxide dismutase activity. Statistically significant differences in MDA were detected in the rats exposed to hypobaric hypoxia followed by reoxygenation, as compared with the control group which was maintained under normoxia conditions and with the rats treated with LA or with vitamin E. Also, we found that the carbonyl proteins, hydrogen donor capacity and SOD activity values were significantly influenced by the LA and vitamin E treatment. The results obtained suggest that the chronic treatment with LA or vitamin E has a protective effect against the oxidative stress associated with an episode of acute hypobaric hypoxia followed by reoxygenation.

P-058

Intracellular signalling involved in oxLDL-induced apoptosis in macrophages: Protective effect of phenolic compounds

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Oxidized LDL (oxLDL) play an important role in the onset of atherosclerosis. Apoptosis has been demonstrated to be the main mechanism for cell death in atherosclerotic plaques. However, the mechanism underlying the apoptotic process is not fully understood yet and poorly investigated. Increased attention has been devoted to phenolic compounds because of their beneficial role in the prevention of atherosclerosis and oxidative stress-associated diseases. The aim of the study was to assess the intracellular signalling pathways involved in the appearance of apoptosis induced by oxLDL in J774.A1 murine macrophages. We also investigated the effects of the two phenolic antioxidants oloeuropein and protocathecuic acid. The occurrence of oxidative stress was evaluated by the cytofluorimetric and spectophotometric determination of ROS and GSH intracellular contents. Apoptosis, mitochondrial membrane potential, caspase-3, -8 and -9 activation were assessed by flow cytometry analyses; p66Shc, BAX, Bcl-XL and Bcl-2 protein expression by western blotting. Cells treated with oxLDL underwent redox imbalance characterized by the irreversible and significant depletion of GSH. Such occurrence was accompanied by the up-regulation of p66Shc, the oxidative stress sensor protein recently identified as a cytoplasmatic signal transducer in oxidative stress-induced apoptosis. As later effects we found an altered ratio between the proapoptotic and anti-apoptotic members of Bcl-2 protein family (increased Bax and reduced Bcl-XL expressions), mitochondrial potential loss and, consequently, apoptosis through caspase-9 and -3 activation. The presence of oleuropein and protocatechuic acid in cultures treated with oxLDL prevented apoptosis by inhibiting redox imbalance and involved signals. Furthermore, the

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phenolic compounds seemed to improve the antioxidant cell defences by increasing the expression of the antioxidant protein Bcl-2.

P-059

Inhibition of leukotriene B_4 production in human neutrophils by 2-styrylchromones

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2-Styrylchromones are a small group of naturally occurring chromones, vinylogues of flavones (2-phenylchromones). Natural and synthetic 2-styrylchromones have been tested in different biological systems, showing activities with potential therapeutic applications. Certain flavones are potent inhibitors of the production of eicosanoids, a group of powerful proinflammatory signalling molecules. However, the anti-inflammatory potential of 2-styrylchromones, concerning their possible interference in this pathway, has not been explored so far. Lipoxygenases (LOXs) are a group of enzymes involved in the arachidonic acid metabolism. From the LOXs existent in the mammalian tissues, 5-lipoxygenase (5-LOX), which is mainly found in cells of myeloid origin, is the most implicated in inflammatory and allergical disorders. 5-LOX produces 5-hydroxyeicosatetraenoic acid (5-HETE) and various leukotrienes (LTA_4-LTE_4), being 5-HETE and LTB_4 potent chemoattractant mediators of inflammation. Thereby, the aim of the present work was to study the putative inhibitory effect of 2-styrylchromones on the production of LTB_4 by human neutrophils. The obtained results show that some of the tested compounds are strong inhibitors of the LTB_4 production, which makes them promising subjects of study for their possible application in the treatment of inflammatory processes.

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P-060

Solid phase extraction of polyphenols with antioxidant activity from herbal infusions

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Polyphenols exist as an extremely diverse number of chemical structures and biological activities. Their structural elucidation and quantification is of great importance in order to find their bioavailability and bioactivity. Extraction of these compounds from aqueous herbal infusions is important, attempting to eliminate accompanying compounds that interfere with their quantification, while concomitantly concentrating them. Extraction methods include liquid–liquid and solid phase (SPE) extraction. In the present work, the capacity of a C18 (6 cm^3; 1 g) Mega Bond Elut cartridge to extract, concentrate and fraction phenolics was tested, using herbal aqueous infusions as starting point. Using 10 mL samples of a standard solution of 17 distinct phenolics, it was possible to resolve them sequentially using water and methanol as eluting solvents. Retrieving 2 mL aliquots of 50% (v/v) aqueous methanol it was possible to separate most of the phenolic compounds: those more polar in nature, e.g. chlorogenic acid, eluted first, those more apolar, e.g. caffeic acid, eluted in the second aliquot and flavonoids, e.g. rutin, eluted in the third aliquot. Extremely apolar compounds, as are phenolics associated with flavour, eluted only with plain methanol. Using the same SPE cartridges it was possible to concentrate all phenolic compounds up to 3-fold in plain methanol. This method of extraction is efficient in concentrating phenolic compounds in aqueous matrices and avoiding resorting to time and reagent consuming methods, as well as drying procedures that may eventually damage the compounds. Further studies include using biological fluids as starting feedstock, e.g. plasma.

P-061

Effects of complex i inhibitors on mitochondrial nitric oxide synthase functional activity

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The selective inhibition of mitochondrial NADH-dehydrogensae activity by pesticides as rotenone and pyridaben may constitute part of the pathogenic mechanism of Parkinson's disease. The Parkinsonian syndrome induced by pesticides is associated with the impairment of mitochondrial function. Rotenone and pyridaben have been recognized as selective inhibitors of mitochondrial complex I activity. Rotenone and pyridaben show a selective inhibition of O_2 uptake and respiratory control in rat brain mitochondria in the presence of NAD-dependent substrates. The IC_{50} of rotenone and pyridaben for complex I inhibition were in the range 1.7–2.2 μM. The determination of NADH-cytochrome c reductase, succinate-cytochrome c reductase and cytochrome oxidase activities in rat brain mitochondrial membranes showed again the selective inhibition of complex I by rotenone and pyridaben. The effect of rotenone and pyridaben in rat brain mitochondria showed an exponential dependence on membrane potential, as determined by Rh-123 fluorescence. The mitochondrial respiration that is affected by supplementation with mtNOS substrates and inhibitors, the mtNOS functional activity, provides an experimental approach to the regulation of mtNOS activity by complex I activity. In rat brain mitochondria, rotenone and pyridaben markedly decreased mtNOS functional activity with NAD-dependent substrates but not when the substrate was succinate. This suggests than the activity of the mtNOS inserted in the inner mitochondrial membrane depends on complex I activity. This regulation and the role of mitochondrial NO diffusion as a signal for mitochondrial biogenesis could have a role in the aetiopathology of Parkinson's disease.

P-062

Effect of closure type on the polyphenol composition, antioxidant activity and vasodilation capacity of a stored red wine

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The inverse correlation between moderate consumption of red wine and incidence of cardiovascular diseases (CD) has been ascribed mainly to its high content of polyphenols, in particular due to its protective effects against low density lipoproteins (LDL) oxidation, and to direct vascular effects, in particular through their abilities to induce endothelium-dependent relaxation. The polyphenol composition of a red wine depends on several factors, including bottle ageing, which is highly dependent on the oxygen barrier properties of closures. Thus, the aim of this work was to evaluate the impact of natural cork vs two synthetic closures on polyphenol composition of a bottled Portuguese red wine, stored during 8 years in similar conditions and the repercussions on either antioxidant activity against free radical-mediated oxidation of isolated human LDL or vasorelaxation capacity in rat isolated aortic rings. Our data indicate that the wine sealed under cork presents significant higher contents in anthocyanins and oligomeric procyanidins, as evaluated by HPLC/DAD, a lower polymerization degree and a lower CIELab parameter H°, indicating a lower degree of oxidation. Such properties are mainly reflected on a higher protection to LDL oxidation, in terms of inhibition periods of conjugated dienes formation. All the wine samples induce endothelium-dependent vasorelaxation via NO-release, but this property does not seem to be so affected by the closure type. In conclusion, cork closure seems to provide enhanced health benefits for the wine consumer, in the context of CD prevention.

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P-063

Cytoprotective properties of α-tocopherol are related to gene regulation in cultured d-galactosamine-treated human hepatocytes

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Vitamin E has demonstrated antioxidant activity and gene regulatory properties. D-galactosamine (D-GalN)-induced cell death is mediated by nitric oxide in hepatocytes and it is associated with hepatic steatosis. The beneficial properties of α-tocopherol and its relation to oxidative stress and gene regulation were assessed in D-GalN-induced cell death. Hepatocytes were isolated from human liver resections by collagenase perfusion technique. α-tocopherol (50 μM) was administered at the advanced stages (10 h) of D-GalN-induced cell death in cultured hepatocytes. Cell death, oxidative stress, α-tocopherol metabolism, NF-kB-, PXR- and PPAR-α-associated gene regulation were estimated in hepatocytes. Transfection studies using CYP3A4 promoter constructions and PXR over-expression vector were carried out in HepG2 cells. D-GalN increased cell death and α-tocopherol metabolism. α-tocopherol exerted a moderate beneficial effect against apoptosis and necrosis induced by D-GalN. Nevertheless, the beneficial property of the vitamer was not related to a reduction of intracellular free radical generation induced by D-GalN in cultured hepatocytes. α-tocopherol increased CYP3A4 promoter activity through PXR sequences in transfected HepG2 cells. The induction (rifampicin) or inhibition (ketoconazole) of α-tocopherol metabolism and the pregnane X receptor (PXR) over-expression showed that the increase of PXR-related CYP3A4 expression by α-tocopherol enhanced cell death in hepatocytes. Nevertheless, the reduction of NF-*k*B activation and inducible nitric oxide synthase (NOS-2) expression and the enhancement of peroxisome proliferator-activated receptor (PPAR-α) and carnitine palmitoyl transferase (CPT1) gene expression by α-tocopherol may be relevant for cell survival. In conclusion, the cytoprotective properties of α-tocopherol are mostly related to gene regulation rather than antioxidant activity in toxin-induced cell death in hepatocytes.

P-064

Singlet oxygen-mediated tryptophan oxidation: Characterization of potential markers of protein oxidation

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Proteins are major targets for oxidative damage due to their abundance and rapid rates of reaction with radicals and excited state species such as singlet oxygen. Exposure of Tyr, Trp and His residues (either free or in proteins) to singlet oxygen generates peroxides in high yield. The structures of some of these peroxides and their breakdown products have been elucidated. Protein peroxides have also been detected on proteins within intact cells on exposure to a photosensitizer and light, but little is known about these peroxides and their decomposition. In this study we have characterized peroxides and breakdown products formed on free Trp, in peptides and on proteins using liquid chromatography-mass spectroscopy (LC-MS). Photo-oxidation of free Trp gives seven major products: two isomeric hydroperoxides, two alcohols, two diols and N-formylkynurenine. These materials are consistent with reactions of singlet oxygen. The hydroperoxides are readily decomposed at elevated temperatures and in the presence of reductants, to give alcohols. With peptides and proteins, the enzyme pronase has been utilised to liberate free amino acids from the parent compound for analysis. Under the conditions employed, some of the products characterized above, plus others, are sufficiently stable to be quantified post-hydrolysis by LC-MS and, hence, can be used as markers of singlet oxygen generation and damage to proteins. The products generated by other oxidants have also been examined and shown to differ in nature or quantity. This approach may allow the quantification of protein modification in intact cells arising from singlet oxygen formation.

P-065

Regulation of the expression of soluble guanylyl cyclase by hydrogen peroxide (H_2O_2)

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Reactive oxygen species (ROS) have been proposed as mediators of tissue damage in different pathophysiological conditions. However, increasing evidence points to hydrogen peroxide (H_2O_2) as an autacoid with well-defined non-toxic effects. Some previous studies have explored the relationship between H_2O_2 and nitric oxide (NO), but the relationships between H_2O_2 and the mechanisms involved in the cellular response to NO have not been extensively explored. The present experiments were designed to analyse the ability of H_2O_2 to modulate the cellular content of the NO receptor, the soluble guanylyl cyclase. Rat aortic smooth muscle cells (RASM) were incubated with H_2O_2 at different times and concentrations. Then we evaluated protein content by Western blot, mRNA expression by Northern blot, cGMP synthesis by radioimmunoanalysis and sGC promoter activity by transfection assays. H_2O_2 increased the sGC β1 protein content in RASM, in a dose- and time-dependent manner (maximum content after 4 h with 10^{-4} M of H_2O_2:182 ± 16% of basal value). As a consequence of this effect, RASM pre-treated with H_2O_2 (4 h with 10^{-4} M) and then incubated with sodium nitroprusside (SNP) showed an increased synthesis of cGMP (178 ± 3%), as well as an increased phosphorylation at Ser-239 of the PKG substrate VASP (214 ± 23%), with respect to control cells incubated with SNP. Neither β1 sGC mRNA expression nor the activity of the ß1 sGC promoter increased in the presence of H_2O_2. In conclusion, these results suggest that H_2O_2 increases the cellular content of sGC by a mechanism independent of changes in mRNA expression. In some pathological conditions in which ROS increases, an increased response to NO could be observed.

P-066

Exhaustive exercise causes a decrease in oxidative stress and an increase in total antioxidant activity of elite triathlete saliva

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Studies on elite triathlete plasma have demonstrated that there is an increase in oxidative stress after triathlon even though triathletes show an increase in total antioxidant activity. However, little is known about the effect of exhaustive exercise on salivary total antioxidant activity and oxidative stress. In order to determine the effect of exhaustive exercise on the production of nitric oxide (NO), oxidative stress (OS), uric acid (UA) and total antioxidant activity (TAA), 12 elite triathletes (men and women) were studied during the Falcon 2006 triathlon. Stimulated saliva samples were withdrawn 1 h, 24 h before and immediately after the triathlon. The production of NO was determined by the Griess reaction, OS (as lipid hydroperoxide concentration) was determined by the FOX method, UA by enzymatic method, and the TAA by ABTS method. On the one hand, exhaustive exercise caused an increase in both salivary UA concentration and TAA immediately after the triathlon. On the other hand, there was a decrease in salivary OS. However, exhaustive exercise did not change the production of NO metabolites. These data suggest that exhaustive exercise causes a decrease in salivary oxidative stress and this result could be explained by the fact that both UA and TAA increased after the triathlon.

P-067

Menadione triggers human *choriocarcinoma* cell death through superoxide generation and glutathione conjugation

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RIGHTSLINK

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The placenta is considered to be the dominant lipid peroxide—and ROS (reactive oxygen species)—producing site during gestation. The aim of the present study was to establish a model of placental oxidative stress in cultured cells in order to explain the roles of oxygen radicals in inducing cells dysfunction. To induce oxidative stress in *choriocarcinoma* cells derived from human syncytiotrophoblast we used the menadione (MEN 2-methyl-1,4-naphtoquinone), which through redox cycling may generate superoxide anion, semiquinone radicals and hydrogen peroxide. Produced by menadione ROS can directly damage macromolecules including DNA, protein and lipid membranes. MEN is also involved in arylation of cellular thiols resulting in depletion of glutathione and inhibition of sulphydryl-dependent proteins. The cell line JAR (ATCC HTB-144) was cultured in the presence of 100 mM MEN for up to 24 h. The intracellular level of $O_2^{\bullet -}$ in JAR cells became increased after MEN treatment reaching the maximum after 4 h, but the level of H_2O_2 produced by MEN was not estimated. Lipid peroxidation products: malondialdehyde and 4-hydroxy-2-nonenal was increased to 6-fold compared to untreated cells, that indicate the $O_2^{\bullet -}$ is a main factor in lipid peroxidation process. In JAR cells treated with MEN we observed an increase of superoxide dismutase activity, but decrease of catalase and glutathione peroxidase activity. MEN induced cytotoxicity is correlated with depletion of glutathione (GSH) by its conjugation with MEN, as well as a loss of ATP. Additionally, we find that MEN inhibited activity of glucose-6-phosphate dehydrogenase and gliceraldehyde-3-phosphate dehydrogenase, key thiol enzymes involved in glutathione and ATP metabolism.

P-068

Assessment of oxidative damage to DNA by flow cytometry and high content bioimaging assays

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Flow cytometry (FCM) and high-content bioimaging analysis (HCA) based on single-cell multiparametric fluorescence measurements allow characterization of heterogeneous cell populations. Miniaturization of such FCM and HCA assays are excellent alternatives for analysis of cytotoxicity *in vitro*. Oxidative damage to DNA is observed *in vitro* during mutagenesis, carcinogenesis and cytotoxicity caused by different agents. 8-oxoguanine (8-oxoG) is a major product of oxidative DNA damage and, therefore, a useful marker of DNA oxidation. The purpose of this work was to provide fast and simple methods for studies of oxidative damage to DNA. We present here complementary FCM and HCA assays we developed for such studies. The assays were performed on hepatic (HepG2), neuronal (SH-SY5Y) and renal (A704) cells growing on 96-well plates and acutely exposed to chemicals or to a combination of methylene blue plus light (positive control). We used the OxyDNA assay (Calbiochem) for detection of oxidative damage to DNA, which is based upon the binding of a FITC-labelled antibody to 8-oxoG epitopes in DNA of fixed cells. For HCA, cells were fixed *in situ*, stained with anti-8-oxoG antibody and nuclei counterstained with DAPI. HCA was performed with the InCell Analyser 1000 (GE Healthcare) using the appropriate algorithms for cell segmentation and data calculation. For FCM, cells remained attached during toxicant exposure and were trypsinized and resuspended for fixation, fluorescent staining and analysis in a 96-well plate-loading flow cytometer (Cytomics FC500 MPL, Beckman-Coulter). We conclude that FCM and HCA provide complementary information and allow a consistent detection and quantification of oxidative effects on DNA.

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P-069

Modulation of calcium regulatory protein Ca-ATPase (SERCA) and possible pharmacological implications

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The maintenance of calcium gradients represents a major energy expediture of cells and is tightly coupled to rates of oxidative phosphorylation and the generation of ROS. Sarco/endoplasmic reticulum (SR/ER) Ca-ATPase (SERCA) transports cytosolic Ca^{2+} into the SR/ER against concentration gradient. SERCA belongs to calcium regulating proteins highly sensitive to oxidative stress and its preferential and selective oxidation at critical sites results in regulation of its function in a reversibile manner. Compounds of high antioxidant activity and protection against pathological reduction of SERCA activity or concentration may represent valuable lead molecules for the development of new drugs, potentially offering dual protection against cardiovascular, skeletal and inflammatory diseases. We found that trolox and the standardized flavonoid extract from leaves of Ginkgo biloba (EGb 761) protected SERCA activity of rabbit skeletal muscles against oxidation by hypochloric acid *in vitro*. On the other hand, Pycnogenol® (Pyc), a standardized flavonoid extract of Pinus pinaster bark, inhibited SERCA activity both in the presence and in the absence of oxidants. Inhibition of SERCA activity by Pyc may lead to an increase of tightly controlled cytosolic Ca^{2+}. A mild increase of cytosolic Ca^{2+} has been suggested to induce adaptive cellular response to oxidative stress by decreasing ROS generation while a higher and long-lasting increase was reported to induce apoptosis and thus anti-tumour effects of Pyc. Pyc may represent a drug with the ability to affect cellular processes known to be regulated by Ca^{2+}.

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P-070

Telomere length regulation and chronic oxidative stress

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Chronic oxidative stress and systemic inflammation play major roles in the pathology of several diseases among which is chronic obstructive pulmonary disease (COPD) [1]. Telomere length has been observed to be negatively associated with increased oxidative stress and inflammation *in vitro* as well as in population studies [2,3]. Our aim was to investigate telomere length in COPD patients and healthy controls in relation with the antioxidant enzyme superoxide dismutase (SOD). In a patient-control study, DNA was isolated from white blood cells of 32 cachectic COPD patients, 63 non-cachectic COPD patients and 19 healthy, age-matched controls. Telomere lengths were measured by quantitative PCR [4]. SOD activity was determined by the method of Sun et al. [5]. Telomere length was significantly shorter in COPD patients than in controls ($p < 0.05$). No significant difference in telomere length was observed between cachectic and non-cachectic patients. In addition, SOD activity was significantly lower in COPD patients than in healthy controls ($p < 0.001$) and telomere length was positively associated with SOD activity ($p < 0.05$). In a multiple regression model, which included gender, age, disease state (patient or control) and smoking behaviour (in packyears), no effect of smoking behaviour was observed, but SOD activity was significantly associated with telomere length. Since telomere length in COPD patients is lower than in healthy controls and also correlates with SOD activity, the oxidant/antioxidant balance seems to affect telomere length. These data confirm the findings of *in vitro* and animal studies, indicating that antioxidant activity of SOD also prevents telomere shortening in humans.

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P-071

Implication of oxidative and endoplasmic reticulum stress in Pick's disease

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Pick's disease (PiD) is a rare progressive frontotemporal dementia (FTD) that accounts for ~2% of all cases of dementias of the elderly. It is characterized by the presence of aggregated tau-protein, also called Pick bodies, that accumulates in the neurons of the affected regions. As the cause of this conditions is still uncertain, we hypothesize the possible implication of oxidative stress and endoplasmic reticulum (ER) stress in this pathology, and we tested this hypothesis by using a case-control approach in samples from brain tissue bank. The stresses were assessed by mass spectrometry and western-blot analyses. Targets of oxidative damage were identified after 2D electrophoresis and MALDI. In samples from frontal cortex, we found evidences of ER stress such as increased IRE1p, EIF2-α phosporylation, which were associated to depletion in ER chaperones (Grp 78 & Grp 94) and to increased ubiquitination. As this ER stress is related to oxidative stress in other neurodegenerative diseases, we evaluated the concentration of the direct oxidation markers aminoadipic and glutamic semi-aldehydes and the lipoxidation marker malondialdehyde-lysine, which increased in the same locations. This oxidative damage targeted antioxidant enzymes and synaptic proteins. Furthermore, as the content of transcription factors responsible for adequate antioxidant responses and mitochondrial biogenesis showed significant decreases in PiD, we conclude that those cells are not able to cope with increased oxidative stress. To sum up, in PiD, there are signs of increased ER and oxidative stress targeting antioxidant enzymes, which may be related to diminished mitochondrial biogenesis and impaired neuroprotective responses.

P-072

Mitochondrial SOD activity in ALS patients

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Several lines of evidence suggest that mitochondrial dysfunction and oxidative stress may be involved in the Amyotrophic Lateral Sclerosis (ALS) pathogenesis and it is not only restricted to the nervous system but also present in other tissues. Recent works demonstrate that a superoxide-dismutase-1 (SOD1) enzyme portion is located in mitochondria. Some data suggest the presence of altered anti-oxidative defence enzymes activities in blood from ALS patients. Nine sporadic-ALS (SALS) patients and nine healthy-controls were analysed for SOD1, SOD2 and aconitase enzymatic activity in mitochondria of platelets, using spectrophotometry. The biochemical data were compared in controls and ALS-patients and with ALS clinical data. Mitochondrial SOD1-activity was lower in ALS-patients than in healthy-controls and much lower in the more serious cases of ALS, related to respiratory insufficiency, malnutrition, low scores in ALS-FRS scale and ageing. In conclusion, it suggests that mitochondrial-SOD1-acitivity could participate in the evolutionary deterioration of SALS.

P-073

The thioredoxin system as drug target for novel tuberculostatics

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Like other pathogens, *Mycobacterium tuberculosis* has to defend itself against the oxidative attack of the host's innate immune response. Since the antioxidant defence system *of M. tuberculosis* differs substantially from that of the mammalian host, its constituents may be regarded as potential drug targets. Particularly the survival and virulence of isoniazid resistant strains are attributed to the peroxiredoxin-type peroxidases alkyl hydroperoxide reductase (AhpC) and thioredoxin peroxidase (TPx), which not only reduce H_2O_2 and a variety of hydroperoxides but also peroxynitrite. However, the most common AhpC reductant in bacteria, the disulphide reductase AhpF, is deleted in *M. tuberculosis*. Instead, AhpC can be reduced by AhpD, a CXXC-motif-containing protein or by one of the mycobacterial thioredoxins, TrxC. TPx is reduced by thioredoxins B and C. Mycobacteria contain three more peroxiredoxins, the 1-C-Prx AhpE, Bcp and BcpB, whose functions are still unknown. Based on cellular abundance of the enzymes involved and their kinetic efficiencies thioredoxin C-dependent hydroperoxide detoxification by TPx appears to be the most important defence mechanism. The uniqueness of the mycobacterial antioxidant defence system certainly offers the chance for selective inhibition to achieve tuberculostasis without affecting the host. Under consideration of the required selectivity and efficiency, inhibition of mycobacterial thioredoxin reductase appears to be the most attractive strategy.

P-074

Design of trypanocidal drugs interfering with the trypanothione system

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The most unique feature of the trypanosomatids is their ability to transform glutathione into the spermidine derivative trypanothione and, in contrast to their mammalian hosts, to use this redox mediator instead of glutathione for the detoxification of hydroperoxides, heavy metals, drugs and hydroxyaldehydes. In *T. brucei* trypanothione synthesis is achieved by trypanothione synthetase (TryS). Even partial suppression of TryS by dsRNA interference is sufficient to impair viability in *T. brucei* and rapidly kills the parasites under mild oxidative stress. TryS, thus, is a validated drug target and TryS inhibition is considered to be a particularly attractive strategy to fight trypanosomal infections. Our mechanistic studies on TryS had suggested that TryS, like glutathione synthetase, belongs to the group of 'ATP-grasp enzymes'. These proteins undergo substantial conformational changes. Similar conformational changes upon binding of ATP or ATP-homologous inhibitors have been reported for protein kinases. In view of this mechanistic analogy, we screened a diversified compound library that had been designed for a protein kinase inhibition strategy and comprised all major types of kinase inhibitors in order to identify lead compounds active against TryS. The screen yielded 20 compounds that reasonably inhibited TryS. Starting from these findings, we initiated a drug design project that provided a novel series of paullones that specifically inhibit TryS with IC50s < 50 nM. As expected for ATP-homologous inhibitors of ATP-grasp proteins, inhibition is neither affected by ATP nor by GSH. Accordingly, these compounds may be considered as promising leads for the development of useful trypanocidal drugs.

P-075

Metabolic demand and redox alteration regulate CuZn- and Mn-superoxide dismutase activities, protein contents and mRNA expressions in rat white adipose tissue

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The effects of cold and alteration in L-arginine/NO producing pathway on the enzyme activity, protein content and mRNA abundance of

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copper, zinc- and manganese superoxide dismutase (CuZnSOD, MnSOD) were studied in retroperitoneal white adipose tissue (RpWAT). Adult rat males divided in three groups: untreated, L-arginine- (2.25%) and N^{ω}-nitro-L-arginine methyl ester- (L-NAME; 0.01%) treated were exposed to cold ($4 \pm 1^{\circ}C$) during 1, 3, 7, 12, 21 and 45 days. Room temperature-maintained rats ($22 \pm 1^{\circ}C$) served as control. In CuZnSOD there was a trend toward a higher activity in all cold-exposed groups compared to control. In contrast, MnSOD activity was significantly increased after 7, 12 and 21 days, but returned to control level on day 45 in the untreated group. However, MnSOD activity was upregulated by L-arginine and L-NAME throughout all periods of cold exposure. Neither cold nor both treatments affect CuZnSOD and MnSOD protein contents. Cold exposure strongly attenuated, whilst L-arginine and L-NAME maintained CuZnSOD mRNA expression on the control level until day 21. However, on day 45 CuZnSOD mRNA was notably reduced in both treated groups. MnSOD mRNA was fully depleted throughout cold exposure in the untreated group. However, L-arginine and L-NAME restored MnSOD mRNA to control at day 12 and 21, i.e. 3, 7, 12 and 21, respectively. This study shows that both redox alteration and metabolic switch induce differential responses of mRNA, protein and activity in RpWAT and indicate the complexity of transcriptional and post-translational regulation of CuZn- and MnSOD.

P-076

Methionine restriction decreases endogenous oxidative molecular damage and increases mitochondrial biogenesis and uncoupling protein 4 in rat brain

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Ageing plays a key role as a risk factor in the development of neurodegenerative diseases. Caloric restriction (CR) mitigates oxidative stress by decreasing the rate of generation of endogenous damage, a mechanism that can contribute to the slowing of ageing rate induced by this intervention. Various facts recently link methionine intake and metabolism to ageing and methionine restriction (MetR) without energy restriction also increases life span, like CR. Therefore, we hypothesized that MetR is responsible for the decrease in endogenous oxidative damage in CR. In this investigation we have subjected male rats to exactly the same dietary protocol of MetR that is known to increase their life span. We have found that, in brain, MetR (a) decreases the mitochondrial complex I content and activity, as well as complex III content, while the complex II and IV and the mitochondrial flavoprotein apoptosis-inducing factor (AIF) are unchanged; (b) increases the mitochondrial biogenesis factor PGC-1α; (c) increases the resistance of brain to metabolic and oxidative stress by increasing mitochondrial UCP4; and (d) decreases mitochondrial oxidative DNA damage and all the five different markers of protein oxidation measured and lowers membrane unsaturation in rat brain. No changes were detected for protein amino acid composition. These MetR-induced beneficial changes, likely derived from metabolic reprogramming at cellular and tissue level, can play a key role in the protection against ageing-associated neurodegenerative disorders.

P-077

Flavonoid oxidation by the immune system: Structure-antioxidant activity of nitrated and chlorinated flavonoids

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In this study the interactions of some flavonoids with pro-inflammatory oxidants hypoclorous acid (HOCl) and peroxynitrite ($ONOO^-$) were analysed, with the aim of identifying the respective products and evaluating the influence of the chemical changes undergone by the flavonoid derivatives on some of their biological properties. From the structural characterization of the products, achieved by UV/Vis spectral analysis, HPLC and ESI/MS/MS, it was found that all flavonoids studied, except naringin, were di-halogenated at C6 and C8, through a typical electrophilic aromatic substitution reaction. From the reaction of flavonoids with $ONOO^-$, several products resulted, including dinitro-derivatives at C3′ and C5′ atoms. The antioxidant activity of the flavonoids under study and of their chlorinated and nitrated derivatives, measured through their $DPPH^{\cdot}$ reducing capacity, showed that while all chlorinated derivatives, except fisetin, present a higher antioxidant activity, nitrated derivatives present a smaller activity than that presented by the parent flavonoids. Their structure-activity relationships were further elucidated, when it was estimated that in chlorinated flavonoids the Cl-substituents decrease the O-H bond dissociation enthalpies of their adjacent hydroxyl groups. In addition, the effect on lipophilicity, determined by their octanol/water partition coefficients, predicted using computational methods, demonstrated that flavonoid chlorides are more lipophilic than the original flavonoids.

P-078

Hyporbromous acid scavenging by flavonoids: Antioxidant activity of bromoflavonoids

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Hypobromous acid, HOBr, is the product of hydrogen peroxide-dependent oxidation of bromide catalysed by eosinophil peroxidase. In the last years its citotoxic activity towards bacteria has been characterized and it has been found out that, in spite of its lower concentrations *in vivo*, not only is HOBr much more reactive than the well-known hypochlorous acid, HOCl, but it also induces damage in a larger extension. In this work the HOBr scavenging activity of flavonoids was analysed, through competition assays, and the products of flavonoid reaction with HOBr characterized by UV/Vis, HPLC and ESI-/MSMS. These studies show that all flavonoids studied, except naringin, were di-brominated on positions 6 and 8 of the A-ring, through an electrophilic aromatic substitution reaction, proceeding through the formation of an intermediate product, the 6-brominated derivative. The antioxidant activity of the brominated flavonoids was evaluated by measuring their $DPPH^{\cdot}$ reducing capacity and it was found that the brominated derivatives present, in general, a higher reducing capacity than the parent flavonoids. Their structure-activity relationships were further elucidated when it was estimated that on brominated flavonoids the Br-substituents decrease the O-H bond dissociation enthalpies of their adjacent hydroxyl groups. In addition, the effect on lipophilicity, determined by their octanol/water partition coefficients, predicted using computational methods, demonstrated that flavonoid chlorides are more lipophilic than the original flavonoids.

P-079

Antiapoptotic role of luminal NADPH in the endoplasmic reticulum in human neutrophil granulocytes

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Genetic deficiency of the endoplasmic reticulum glucose-6-phosphate transporter results in neutropenia and neutrophil granulocyte dysfunction in type 1b glycogen storage disease. Experimental inhibition of the transporter resulted in an increased apoptosis in differentiated HL-60 cells and human neutrophils; the effect could be prevented by NADPH oxidase inhibitor or antioxidants. It was supposed that microsomal glucose-6-phosphate transport has a role in the antioxidant protection of neutrophils, possibly through the substrate supply of the intra-luminal NADPH generating hexose-6-phosphate dehydrogenase. To confirm this hypothesis in this study we have demonstrated the expression of hexose-6-phosphate dehydrogenase in human neutrophils. The presence and activity of the enzyme were shown in the microsomal fraction of the cells. The expression and activity of 11β-hydroxysteroid dehydrogenase type 1, another NADP(H)-dependent microsomal enzyme responsible for cortisone-cortisol interconversion, were also detected in human neutrophils. The NADPH-generating cortisol dehydrogenase activity of the enzyme prevented neutrophil apoptosis provoked by the inhibition of the glucose-6-phosphate transporter. In conclusion, the maintenance of the luminal NADPH pool is an important anti-apoptotic factor in neutrophil granulocytes.

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P-080

Peroxynitrite causes fragmentation of extracellular matrix

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The extracellular matrix, a complex structure comprising of proteins and proteoglycans, provides strength and elasticity to mammalian tissues and plays a role in the regulation of cellular behaviour. Damage to vascular matrix components is an important factor in the development of atherosclerosis and other diseases. Peroxynitrite/peroxynitrous acid ($ONOO^-$/ONOOH), generated by reaction of nitric oxide ($^{\bullet}NO$) with superoxide ($O_2^{-\bullet}$), has been implicated in such damage, though little is known about how this occurs. We have previously shown that $ONOO^-$/ONOOH fragments isolated glycosaminoglycans via a hydroxyl radical-like mechanism, resulting in the formation of specific fragments in a concentration-dependent manner [1]. In this study the reaction of authentic $ONOO^-$/ONOOH and that generated by the decomposition of 3-(4-morpholinyl)-sydnonimine (SIN-1), with matrix isolated from the vascular smooth muscle cell line A7r5 and porcine thoracic aorta was examined. Authentic $ONOO^-$/ONOOH causes the release of glycosaminoglycan and protein fragments from vascular smooth muscle cell- and porcine thoracic aorta-derived matrix in a concentration dependent manner. This release is modulated by the local pH and the presence of bicarbonate. SIN-1 causes the release of glycosaminoglycan, but not protein, from vascular smooth muscle cell-derived matrix in a concentration-, time- and pH-dependent manner. Pig aorta matrix was found to be resistant to damage by SIN-1, with no observed release of glycosaminoglycan or protein under the experimental conditions. The data presented here suggest that the formation and reactions of ONOOH may, at least in part, be responsible for damage to the extracellular matrix at sites of inflammation.

Reference

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P-081

Activation of the GPx2 promoter by β-catenin

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GPx2, the gastrointestinal glutathione peroxidase, is a selenoprotein predominantly but not exclusively expressed in the intestine. It is highly expressed in the proliferative area of the intestinal crypt-to-villus axis and in Paneth cells. Additionally, GPx2 is up-regulated during development of gastrointestinal adenocarcinomas with the highest expression in early stages of malignancy. Since both normal proliferation and differentiation of intestinal epithelial cells and carcinogenesis are regulated by the Wnt pathway it was tested whether GPx2 might be a target of the β-catenin/TCF complex which transfers Wnt signals. The GPx2 promoter contains five putative β-catenin/TCF binding sites. Accordingly, the promoter was active in two cell lines in which β-catenin was constitutively active, HepG2 and SW480, but not in BHK-21 cells in which the Wnt pathway is silent. Transfection of β-catenin/TCF activated the GPx2 promoter in all three cell lines. Over-expression of wild-type APC in SW480 cells which harbour an APC gene with a defect in the β-catenin binding site inhibited GPx2 promoter activity. Truncation of the promoter identified one β-catenin/TCF binding site that was sufficient for activation. Mutation of this site reduced the response to β-catenin/TCF by 50%. These findings suggest a function of GPx2 in the maintenance of normal renewal of the intestinal epithelium. Whether its up-regulation during carcinogenesis supports tumour growth or can rather be considered as counteracting remains to be investigated.

P-082

Influence of age and castration on pro-antioxidant and pro-inflammatory processes in liver from female rats. Preventive effect of growth hormone, melatonin and oestrogens

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It is a general agreement that, besides free radical production, ageing is a process also related to inflammation. The aim of the present study was to investigate oxidative stress and inflammation processes in liver obtained from old female rats and the influence of chronic administration of the above mentioned hormones. Sixty-one 22-month old Wistar female rats were used. Intact animals were divided into three groups and treated for 10 weeks with GH, melatonin or saline and 40 ovariectomized animals were divided into five groups and treated for the same time with GH, melatonin, oestrogens, phytoestrogens or saline. A group of 2-month old female rats was used as young control. Protein expression of iNOS, HO-1, IL-6, TNF-α and IL-1β were determined by Western blot analysis. The activity of glutathione peroxidase (GPx), glutathione-S-transferase (GST) and the levels of NO, LPO, TNF-α, IL-1β and cytochrome *C* were determined in different fractions of liver. The results show the presence of an age-dependent increase in TNF-α, IL-1β level and lipid peroxidation products, with maximal values in the castrated females. Ageing and castration increased expression levels of pro-inflammatory cytokines, HO-1 and iNOS. Administration of GH, melatonin, Eos and Phyt to the ovariectomized groups significantly increased GPx and GST activity and decreased NO level in the mitochondrial fraction as compared with untreated rats. A significant increase in cytochrome *C* in the mitochondrial fraction and a decrease in the cytosol fraction together with all pro-inflammatory substances was also found with all treatments. Oxidative stress and inflammation induced by ageing are more marked in castrated than in intact females. Administration of the different hormonal replacement therapies reduces both situations.

P-083

Iron and copper mediated DNA damage and apoptosis induced by 6-hydroxydopamine

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6-Hydroxydopamine (6-OHDA) is one of the commonly used neurotoxins to produce animal model of Parkinson's disease. To clarify the mechanism of 6-OHDA neurotoxicity, we examine the apoptosis and oxidative DNA damage induced by 6-OHDA using human neuroblastoma SH-SY5Y cells. 6-OHDA induced the DNA ladder formation, characteristic for apoptosis, in SH-SY5Y cells. 8-Oxo-7, 8-dihydro-2′-deoxyguanosine (8-oxodG), a biomarker of oxidative DNA damage, was increased in SH-SY5Y cells treated with 6-OHDA. 6-OHDA-induced apoptosis and 8-oxodG formation were inhibited by an iron chelator or a Cu(I) chelator. Furthermore, the formation of 8-oxodG is observed in isolated calf thymus DNA incubated with 6-OHDA plus Fe(III) complex or Cu(II). In addition, we examined 6-OHDA-induced DNA damage in the presence of Fe(III)EDTA or Cu(II) using ^{32}P-5′-end-labelled DNA fragments. 6-OHDA induced DNA damage in the presence of Fe(III)EDTA or Cu(II). Fe(III)EDTA-mediated DNA damage inhibited by free hydroxyl radical (•OH) scavengers suggested that the DNA damage is mainly due to •OH generated via the Fenton reaction. In the presence of Fe(III)EDTA, 6-OHDA caused DNA damage at every nucleotide. In contrast, •OH scavengers did not inhibited Cu(II)-mediated DNA damage. Methional, which scavenges species with weaker reactivity than •OH, inhibited the DNA damage, indicating that reactive oxygen species such as Cu(I)OOH may participate in Cu(II)-mediated DNA damage. DNA damage induced by 6-OHDA plus Cu(II) occurred frequently at thymine and cytosine residues. We concluded that metal-mediated DNA damage may play an important role in 6-OHDA-induced dopaminergic neuronal cell death, resulting in Parkinsonism.

P-084

Thioredoxin system of *Streptomyces coelicolor A3(2)*

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Despite the highly oxidizing environment (21% oxygen, at sea level), at normal conditions, the cell cytoplasm of all aerobic organisms are kept reduced and proteins contain free sulphydryl groups. In the cytoplasm were identificated two major systems responsible for maintaining a reduced state: thioredoxin and glutathione/glutaredoxin system. *Streptomyces coelicolor A3(2)* is genetically the most studied member of streptomycetes and it also can serve as a suitable model organism to study thioredoxin system, because it lacks a cooperating glutatione/glutaredoxin system. In most organisms are multiple functions accomplished by single thioredoxin. The complete genome sequence of *S. coelicolor* revealed several possible genes: three for thioredoxins, other two for putative thioredoxins, one for thioredoxin reductase and two genes for putative thioredoxin reductases that are involved in unknown biological processes. It seems that *S. coelicolor* has a very complex redox system, which can respond to complex multicellular development of streptomycetes. The crystal structure of thioredoxin A has been determined at 1.5 Å resolution using a synchrotron-radiation source. The protein reveals a thioredoxin-like fold with a typical CXXC active site. The crystal exhibits the symetry of space group $P2_12_12$, with unit-cell parameters a = 43.6, b = 71.8, c = 33.2 Å.

P-085

Phenylpropanoids as a new class of plant-derived antioxidants feasible for skin diseases. What we learn from plant defense

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Phenylpropanoids (PPs) belong to the largest group of secondary metabolites produced by plants, mainly in response to biotic or abiotic stresses such as infections, wounding, UV irradiation and exposure to ozone, pollutants and other hostile environmental conditions. PPs are synthesized from aminoacids and carbohydrates on the stress-induced enzymes through common biosynthetic shikimic acid pathway. It is thought that the molecular basis for the protective action of PPs in plants is their antioxidant and free radical scavenging properties. PPs are parent molecules for practically all classes of plant polyphenols such as flavonoids, isoflavonoids, tannins, lignins, glycosides, coumarines, etc. These numerous polyphenolic compounds are major biologically active components of human diet, spices, aromas, wines, beer, essential oils, propolis and traditional medicine. Over the last few years, much interest has been attracted to natural, biotechnologically produced and synthetic PPs for medicinal use as antioxidants, UV screens, cardio-protective, chemo-preventive, anti-tumour, anti-virus, anti-inflammatory, wound healing and anti-bacterial agents. Learning from plants, free radical-driven, molecular and cellular processes modulated by selected glycosilated PPs produced biotechnologically in the cultures of medicinal plant cells were studied in human reconstructed epidermis, primary keratinocyte and fibroblast cultures as well as in the *in vivo* models of skin pathologies (UV-induced damage, aseptic inflammation and wound healing).

P-086

Metal complexes of plant polyphenols: Free radical scavenging properties and protection against free radical-mediated cell and tissue damage

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Interaction of various plants polyphenols with transition metals leads to the formation of corresponding metal complexes. It was found that complexes of transition metals (Fe^{2+}, Fe^{3+}, Cu^{2+}) with rutin, taxifolin, (−)-epicatechin, luteolin, but not with verbascoside possess remarkable superoxide dismuting activity. As a result, the superoxide scavenging properties of the flavonoid-metal complexes were significantly higher than those of the parent flavonoids. Moreover, iron (II) bound to rutin or verbascoside found a relatively poor Fenton catalyst in comparison with copper (II) in complex with the same ligands. In addition, it was shown that hydroxyl radicals produced as a result of homolytic cleavage of hydrogen peroxide by the metal-flavonoid complexes were effectively scavenged by the flavonoid ligand moiety in a site-specific manner. In conclusion, polyphenol-metal complexes were found to be effective scavengers of superoxide anions, which were also able to safely decompose hydrogen peroxide. Possible therapeutic benefits of metal complexes with plant polyphenols were demonstrated in several *in vitro* and *in vivo* models of ROS-mediated cellular and tissue damage.

P-087

Hepatic iron content corresponds to the PBL sensitivity to oxidative stress

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The piglet is born with limited iron supplies. If not supplemented, piglets dramatically lose their body iron stores during the first days of post-natal life. The aim of this study was to investigate the influence of hepatic iron content on susceptibility of blood cells to oxidative stress. PBL from newborn and 7-day old piglets were isolated by density gradient centrifugation on Histopaque 1007, resuspended in RPMI1640 medium supplemented with 20% FCS and treated with the DNA damaging agent: X-rays (dose 0–5 Gy, 200 kV, 5 mA, dose rate 1.2 Gy/min) on ice, H_2O_2 (0–250 μM), tert-butyl hydroperoxide (0–500 μM) or NaOCl (0–1000 μM) in PBS pH 7.4 for 15 min at 4°C. The extent of DNA damage was estimated by alkaline comet assay. As expected, iron body stores of non-supplemented animals decrease significantly during the first 4 days of life. Interestingly, DNA damage induced by H_2O_2, X-radiation, NaOCl or tert-butyl hydroxide in lymphocytes taken from newborn piglets was significantly higher than in those taken from 7-day old animals. However, no difference was found between untreated lymphocytes, despite hepatic iron content of donor animals. Our data show that decreased hepatic iron content correlates with decreased susceptibility of blood lymphocytes to oxidative stressors. However, lack of differences between untreated cells indicates that hepatic iron content had no effect on blood lymphocytes in the normal, non-pathological condition.

P-088

Regulation of ionizing radiation-induced apoptosis by mitochondrial $NADP^+$-dependent isocitrate dehydrogenase

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Ionizing radiation induces the production of reactive oxygen species, which play an important causative role in apoptotic cell death. Recently, we demonstrated that the control of mitochondrial redox balance and the cellular defense against oxidative damage is one of the primary functions of mitochondrial $NADP^+$-dependent isocitrate dehydrogenase (IDPm) by supplying NADPH for antioxidant systems. In this report, we demonstrate that modulation of IDPm activity in U937 cells regulates ionizing radiation-induced apoptosis. When we examined the regulatory role of IDPm against ionizing radiation-induced apoptosis in U937 cells transfected with the cDNA for mouse IDPm in sense and antisense orientations, a clear inverse relationship was observed between the amount of IDPm expressed in target cells and their susceptibility to apoptosis. Upon exposure to 2 Gy of gamma-irradiation, there was a distinct difference between the IDPm transfectant cells in regard to morphological evidence of apoptosis, DNA fragmentation, cellular redox status, oxidative damage to cells, mitochondria function and the modulation of apoptotic marker proteins. In addition, transfection of HeLa cells with an IDPm small interfering RNA (siRNA) decreased activity of IDPm, enhancing the susceptibility of radiation-induced apoptosis. Taken together, these results indicate that IDPm may play an important role in regulating the apoptosis induced by ionizing radiation and the effect of IDPm siRNA on HeLa cells offers the possibility of developing a modifier of radiation therapy.

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P-089

Differential intracellular toxicity of b-amyloid peptide between cell lines

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The risk of developing cancer is lower among patients with Alzheimer's Disease than in non-demented, age-matched patients. Conversely, risk of developing Alzheimer's Disease may be less for patients with a history of cancer [1]. There is growing evidence of intra-cellular toxicity of β-amyloid peptide. Its intra-cellular accumulation is facilitated by ABAD (beta-amyloid biding alcohol dehydrogenase) and causes mitochondrial dysfunction and apoptotic death [2]. In our previous studies we showed that beta-amyloid causes an increased rate of peroxide production by mitochondria, increased aggregation of brain mitochondria, decreased lysosomal content in cells probably due to lysosomal rupture and apoptosis preceded by cytochrome C release by mitochondria. The aim of this study was to examine the possibility that β-amyloid causes different levels of damage in different cell types (proliferative cell line, cancer cell line and cell in primary culture). We measured oxidative stress markers, apoptosis markers and cellular viability in a human cancer cell line (MCF7), in a mouse cancer cell line (B16), in primary cultured neurons from foetal rat brain, in primary cultured fibroblast from foetal rat (REFs), in differentiated and non-differentiated PC12 cells and in lymphocytes from human blood. Our results show that in general beta-amyloid peptide causes more toxicity to cancer cell lines than in primary cultured cells.

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P-090

Females have higher brain glucose consumption than males and it decays with ageing

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Small-animal PET is acquiring importance for pre-clinical and follow-up studies. Determining the radiotracer normal distribution and quantification are essential for studying physiopatolological alterations, such as cancer. Females live longer than males in many species, including humans. The aim of our study was double: first to determine if brain glucose consumption decreases with age and secondly to establish if there is any difference on brain glucose consumption between male and female mice. We used male or female SAMP mice aged 7 or 20 months. PET scan was performed after fasting for 8–12 h and the brain ^{18}F-FDG uptake was measured under isoflurane anaesthesia. The brain consumption of glucose was evaluated *in vivo* with an Albira PET during 60 min (dynamic scan) and 2×10 min (static scan) after 5.8–11.1 MBq of ^{18}F-FDG intra-peritoneal injection. A whole brain VOI was constructed around the total surface of brain and the images were reconstructed with 3-D MLEM. For each scan VOIs were drawn on brain and the%ID/g were calculated. In 7 month old mice, the FDG uptake was significantly higher in females than in males (0.92 ± 0.03%ID/g vs 0.68 ± 0.02%ID/g, $p < 0.001$). In 20 month old mice the FDG uptake falled significantly compared to the 7 month old mice in both male (1.7-fold, $p < 0.05$) and female (2.6-fold, $p < 0.05$) mice. The different consumption of glucose between genders in old animals was lost, probably because of the loss of oestrogen cycles in females. In conclusion, the FDG uptake of whole brain decreases with ageing and in young animals female brain consumption is higher than brain male consumption.

P-091

Regional nitric oxide concentration dynamics in the hippocampus of living rat evoked by stimulation of glutamate receptors

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Nitric oxide ($^{\bullet}$NO) is a radical molecule that acts as a ubiquitous intercellular messenger. In the hippocampus, $^{\bullet}$NO participates in the mechanisms of synaptic plasticity but neurotoxic actions of $^{\bullet}$NO have also been documented. Both events involve the stimulation of ionotropic glutamate receptors, which trigger a transient increase in $^{\bullet}$NO produced by the neuronal isoform of nitric oxide synthase. Thus, the regulation of glutamate receptor-nitric oxide synthase pathway and the $^{\bullet}$NO profile of change are important determinants of the physiological and pathological $^{\bullet}$NO actions in the hippocampus. The direct measurement of $^{\bullet}$NO in brain tissue *in vivo* and in real time is critical to know its concentration dynamics and bioactivity; however, it is a demanding technological challenge because of low concentration and short half-life of $^{\bullet}$NO *in vivo*. Using electrochemical techniques coupled with selective microelectrodes inserted into the hippocampus of the living rat we have characterized the real time $^{\bullet}$NO production functionally dependent on the stimulation of glutamate NMDA receptors. Once we had established a pattern of $^{\bullet}$NO production in response to sequential stimuli by local injection of glutamate, we observed that a reproducible pattern of response was only achieved for $^{\bullet}$NO concentrations below a few hundred nanomolar, suggesting such a border for $^{\bullet}$NO toxicity. The modulation of $^{\bullet}$NO production was carried out by the local application and systemic administration of both NMDA receptors antagonists (D-AP5 and dizocilpine) and AMPA receptors antagonist (NBQX), as well as application of nitric oxide synthase inhibitors. Evidence suggest a regional-specific $^{\bullet}$NO concentration dynamics along the major neuronal circuit in the hippocampus, encompassing CA1, CA3 and dentate gyrus sub-regions, pointing to distinct regulatory pathways and biological activities for $^{\bullet}$NO along the trysinaptic loop.

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P-092

Progression of electron transport system abnormal fibres with age: COX^-/SDH^N fibres are potential precursors of COX^-/SDH^{++}

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Mitochondrial DNA (mtDNA) deletion mutations and electron transport system (ETS) abnormalities have been implicated in ageing of skeletal muscle. ETS abnormalities are likely caused by mtDNA deletion mutations. Two primary ETS abnormal phenotypes accumulate in skeletal muscle with age: cytochrome c oxidase negative/succinate dehydrogenase normal (COX^-/SDH^N) and cytochrome c oxidase negative/succinate dehydrogenase hyperreactive (COX^-/SDH^{++}). The focus of this study is the cellular and molecular characterization of these phenotypes. Three quadriceps muscles from Fisher 344xBrown Norway rats were analysed for the presence of ETS abnormal fibres at 3-month intervals between 12–39 months. Both phenotypes significantly increased with age. COX^-/SDH^N fibres, however, appeared earlier (12 months), were shorter and were less prone to intra-fibre atrophy than COX^-/SDH^{++} (27 months). We have previously found mtDNA deletion mutations to be associated with COX^-/SDH^{++} fibres. In this study we quantified the intracellular abundance of mtDNA-deletion mutations in COX^-/SDH^{++} fibres and found deletion mutations to accumulate to > 90% of the total mtDNA within ETS-abnormal regions. We also analysed the genotype of 46 COX^-/SDH^N fibres and found that COX^-/SDH^N fibres from 18–24-month-old rats did not have mtDNA deletion mutations, while 85% of COX^-/SDH^N fibres from 27–36-month-old animals had mtDNA deletions. These findings suggest that there are two different groups of COX^-/SDH^N fibres, (i) one that is associated with mtDNA deletion mutations and is a precursor of COX^-/SDH^{++} and (ii) one not caused by mtDNA deletion mutations.

P-093

Effects of 872 MHz radiofrequency radiation on intracellular reactive oxygen species production and DNA damage

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The aim of this study was to examine effects of 872 MHz radio-frequency radiation (RFR) on intracellular production of reactive oxygen species (ROS) and DNA damage. Menadione (K-vitamin) was used as a co-exposure chemical for inducing intracellular reactive oxygen species (ROS) and DNA damage via increased ROS production. Intracellular ROS production was measured by using fluorescent probe 2′,7′-dichlorofluorescein-diacetate (DCFH-DA). The single cell gel (SCG) electrophoresis assay, also known as Comet assay, was used for quantifying DNA-damage. Human neuroblastoma (SH-SY5Y) cells were treated with 25 and 50 μM menadione in the DNA damage and ROS production experiments, respectively. The exposures to 872 MHz continuous wave (CW) or GSM modulated (modulation frequency 217 Hz) RFR were done in four groups; (1) sham (control), (2) RFR, (3) menadione and (4) menadione & RFR exposed. The exposure time was 1 h and specific absorption rate 5 W/kg for both RFR signals. When a CW signal was used, increased DNA breakage ($p < 0.001$) was observed in group 4 compared to group 3. Also a slight, statistically not significant, increase in ROS levels was found with the same exposure protocol, which might explain the observed increase in DNA damage.

P-094

Nitric oxide suppresses the oxidative phosphoryaltion in gastric cancer cell lines

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Dinitrosyl iron complexes (DNIC) are formed under physiological conditions where NO is generated in the presence of iron-sulphur clusters. Since iron-sulphur cluster is an essential component of mitochondrial enzymes such as aconitase in aerobic respiratory chains, the presence of DNIC indicates the impaired mitochondrial oxidative phosphorylation, which is reportedly associated with carcinogenesis. We thus hypothesized that cancer cells may exhibit both increased formation of DNIC and impaired oxidative phosphorylation. Therefore we examined intracellular NO concentrations, the electron paramagnetic resonance (EPR) spectra of DNIC, the oxygen consumption and the lactic acid production in gastric cancer cells compared with normal gastric mucosal cells. Gastric cancer-derived MKN-45 and AGS cells, gastric mucosa-derived RGM-1 cells and its cancer-like transformed RGK-1 cells were examined. Intracellular NO concentrations, the amount of oxygen consumption and lactic acid production were measured with a fluorescence probe DAF2-DA, a dissolved O_2 monitor and an enzymatic L-lactic acid-analysis kit, respectively. We also investigated the DNIC formation in mitochondrial fraction of the cells with EPR. Compared with gastric mucosal cells, cancer cells exhibited increased NO concentrations, decreased oxygen consumption and increased lactic acid production. EPR analysis confirmed the presence of DNIC in cancer cells, not in gastric mucosal cells. Our results suggested that increased intrinsic NO, probably as a result of carcinogenesis, induces the DNIC formation in mitochondria and consequently suppresses the mitochondrial oxidative phospholyration in cancer cells.

P-095

Analytical methods for *in vitro* determination of antioxidant/radical scavenging capacity: From batch assays to automatic flow systems

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During the past decade, the research focused on analytical methodologies for the assessment of antioxidant/radical scavenging capacity in food, botanical and pharmaceutical samples has grown tremendously, especially after the implication of free radical redox-reactions in the pathogenesis of several human diseases [1]. This situation demands the development of fast, robust and reliable analytical methods for routine analysis. Nevertheless, the implementation of this type of assay may be difficult, considering that short-living reactive species are involved and that reproducible reaction conditions are required. These limiting aspects can be overcome by automation. In this context, flow injection analysis and its predecessor computer-controlled techniques have proven to be a valuable tool to improve batch methodologies, mainly due to the versatility, capacity to accommodate a wide variety of assays and high sampling rate. Furthermore, the strict control of reaction time and the reproducible contact between oxidant and antioxidant/scavenger molecules is also achieved. In the present communication, the automation of widely used batch assays is described. 2,2-Diphenyl-1-picrylhydrazyl (DPPH•) [2], Folin-Ciocalteu [3,4] and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) radical cation (ABTS•+) [4] were implemented, exploiting multi-syringe flow injection analysis. Moreover, the *in vitro* determination of hypochlorous acid scavenging capacity [5], under pH and concentration conditions similar to those found *in vivo*, is also presented. Food and pharmaceutical samples were analysed by the proposed automatic methods and results were similar to those obtained by batch methods or to values reported in the literature.

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P-096

Rat and human liver xanthine oxidase and xanthine dehydrogenase: NADH oxidase activity

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To characterize the NADH oxidase activity of both xanthine dehydrogenase (XD) and xanthine oxidase (XO) forms of rat liver xanthine oxidoreductase (XOR) and to evaluate the potential role of this mammalian enzyme as an $O_2^{\bullet -}$ source, kinetic and EPR spectroscopic studies were performed. A steady-state kinetic study of XD showed that it catalyses NADH oxidation, leading to the formation of 1 $O_2^{\bullet -}$ molecule and 1/2 H_2O_2 molecule per NADH molecule, at rates three times of those observed for XO (29.2 ± 1.6 and 9.38 ± 0.31 min^{-1}, respectively). EPR spectra of NADH-reduced XD and XO were qualitatively similar, but they were quantitatively quite different. While NADH efficiently reduced XD, only a great excess of NADH reduced XO. In agreement with reductive titration data, the XD specificity constant for NADH (8.73 ± 1.36 $\mu M^{-1}min^{-1}$) was found to be higher than that of the XO constant (1.07 ± 0.09 $\mu M^{-1}min^{-1}$). It was confirmed that, for the reducing substrate xanthine, rat liver XD is also a better $O_2^{\bullet -}$ source than XO. These data show that the dehydrogenase form of liver XOR is, thus, intrinsically more efficient at generating $O_2^{\bullet -}$ than the oxidase form, independently of the reducing substrate. Most importantly, for comparative purposes, human liver xanthine oxidase activity towards NADH oxidation was also studied and the kinetic parameters obtained were found to be very similar to those of the XO form of rat liver XOR, foreseeing potential applications of rat liver XOR as a model of the human liver enzyme.

P-097

Role of ascorbate in the oxidative protein folding *in vivo*

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It has been described previously that ascorbate participate in the oxidative protein folding *in vitro*, inasmuch as the addition of ascorbate results in disulphide-bond formation in endoplasmic reticulum (ER)-derived liver microsomal vesicles. To investigate if this mechanism also plays a role in the *in vivo* protein folding, we used guinea pigs kept on an ascorbate free diet. After a 2-week-long ascorbate free diet, ascorbate was not detectable in the liver of guinea pigs, so the diet was apparently effective. Beside the development of scurvy, we could detect the induction of main ER chaperons and foldases, such as Grp78, Grp94, and protein disulphide isomerase. These findings indicate ER stress, which is caused by the accumulation of misfolded proteins in the lumen of the ER. We also found increased apoptosis in liver of guinea pigs with scurvy on the 3rd and 4th week of diet. This correlate to the fact that prolonged ER stress can cause apoptosis in the affected cells. We also investigated the lipid peroxidation in guinea pig livers and found elevated TBARS levels on the 4th week of diet, thus the oxidative damage appeared only at the tale-end of the vitamin C withdrawal. These results suggest that persistent ascorbate deficiency leads to ER stress and apoptosis in liver, suggesting that ascorbate contributes to the proper protein folding also *in vivo*.

P-098

Glutathione redox status as an oxidative stress marker in chronic Hepatitis C: Association with lipid metabolism dysregulation and insulin resistance

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Oxidative stress and increased iron storage are common biological features in chronic hepatitis C (CHC). Glutathione (GSH) plays an important role in the defence against oxidative stress induced cell injury. Cell ability to maintain GSH levels is highly important for integrity and cellular function. The GSH (reduced form)/GSSG (oxidized GSH) ratio is a sensitive oxidative stress marker. GSH redox status regulates several cellular signalling processes, such as activation of transcriptional factors whose products mediate inflammatory responses. The objective was to investigate oxidative stress condition in CHC through determination of plasma GSH levels and GSH/GSSG ratio and possible correlation with insulin resistance and lipid metabolism biochemical parameters. Two hundred and fifty-four CHC patients (41.1 ± 12.6 years) and 65 controls were studied. Total GSH levels were determined by a fluorimetric assay; lipid profile (cholesterol, triglycerides, HDL, LDL, apolipoprotein A and B), leptin, insulin and glucose levels (HOMA) were quantified by standard techniques. Plasma GSH levels and GSH/GSSG ratios were significantly reduced in the CHC group compared to controls (19.8 ± 10.5 vs 36.7 ± 12.8; $p = 0.000$ and 5.1 ± 2.7 vs 8.3 ± 4.8; $p = 0.000$). No correlation was found with viral load, body mass index and HOMA. GSH/GSSG ratio showed a negative correlation with LDL/HDL ratio ($R = -0.359$), a predictive parameter of atherosclerosis. In conclusion, oxidative stress is a feature present in chronic hepatitis C, clearly marked by reduced GSH levels and GSH/GSSG ratios. Since 50% of liver cells are endothelial, the association between increased LDL and GSH oxidation also can contribute to systemic vascular dysfunction, atherosclerotic lesion and liver injury in this particular virus infection.

P-099

Manganese complexes of porphyrins as important antiapoptotic compounds: Is hemin the endogenous substrate for erythrocyte transmembrane oxidoredutase?

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Synthetic manganese complexes of porphyrins (ACS) are redox active metal compounds that are employed as SOD and catalase mimetics. These compounds can interact with human erythrocyte membrane acting as external acceptors of transmembrane oxidoredutase (TMOR) activity. TMOR transfers electrons from cytosolic NADH to external oxidants, like ferricyanide, through pathways linked to metabolic regulation and acting as a transducing system of anti-apoptotic and proliferation processes. The Fe^{3+} oxidation product of heme is termed hemin. We propose that hemin resultant from hemoproteins degradation could be an endogenous substrate for TMOR. The aim was to evaluate TMOR activity in the presence of different ACS synthesized in one of our laboratories and hemin. TMOR activity was assayed spectrophotometrically at 535 nm by 3.3×10^{-3} M $FeCl_3$ reduction, forming a complex with bathophenanthrolinedisulphonic acid and the associated reduction of external ferricyanide to ferrocyanide in erythrocyte membrane. ACS compounds and hemin were added 30 min before ferricyanide addition. All assayed compounds, including hemin, shown the capacity to interact with erythrocyte membrane, resulting in a significant decrease of TMOR activity evaluated by decreased reduction of external ferricyanide. The ACS porphyrin synthesized without manganese in the heme core (control) show no interaction with TMOR. The manganese core of these compounds may play an important role in the action of the compounds as electronic acceptors. The interaction between these compounds and the oxidoreductase system of cellular membrane may be associated with anti-apoptotic and proliferation functions. Hemin can be considered as an endogenous substrate for TMOR activity.

P-100

Decreased plasma glutathione levels and glutathione S-transferase activity in patients with chronic renal failure: Possible association with inflammatory process

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Chronic renal failure (CRF) is a pathologic condition associated with vascular disease. Oxidative stress and nitric oxide availability are mediators of vascular injury and inflammation. Glutathione (GSH) is an important antioxidant and an essential cofactor for enzymes like glutathione S-transferase (GST). GSH depletion determines the vulnerability to oxidative attack. The ratio between GSH (reduced form) and GSSG (oxidized GSH) is a sensitive oxidative stress marker. GSH redox status is critical for various biological events as modulation of redox-regulated signal transduction, regulation of cell proliferation and inflammation. GSTs are mainly responsible for metabolization of lipoperoxidation products, like MDA and 4-hydroxynonenal. Plasma GST and erythrocyte nitric oxide synthase (NOS) activities were evaluated by spectrophotometry in 79 patients. Plasma GSH quantification (oxidized and reduced forms) was achieved by fluorimetry. Plasma TNF α, a pro-inflammatory cytokine, was determined using a commercial Elisa Kit. GST was reduced in CRF compared to 70 healthy donors (0.039 ± 0.01 vs 0.138 ± 0.004 U/mL, $p < 0.001$). Although the GSH levels were significantly increased in CRF, the GSH/GSSG ratio presented a marked decrease in this pathology ($p < 0.001$). GST activity directly depends on the GSH/GSSG ratio ($R = 0.280$, $p = 0.012$). TNF α activation is probably related with redox cell status, since reduced GSH/GSSG ratios are associated with increased levels of this cytokine ($R = -0.198$, $p = 0.081$). Decreased GSH/GSSG ratios confirmed the involvement of oxidative stress in CRF physiopathology. In addition, reduced GST activity can be a susceptibility factor for the metabolization of lipoperoxidation products.

P-101

The importance of oxygen conditions in Fanconi anaemia primary fibroblasts cell lines

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At least 11 genes have been identified in relation to Fanconi anaemia (FA) and have been found to form a multinuclear complex in response to oxidative stress [1]. Mutation in these genes leads to defective DNA repair and an increase in 8-oxoG, which is a hallmark of the disease. A failure to detoxify reactive oxygen species (ROS) has been reported in FA cells [2] and various redox abnormalities and *in vivo* pro-oxidant states have been well documented in FA patients [3]. We have compared the oxidative stress parameters and cell growth in human FA fibroblasts: FA-D2 (FANCD2 mutant)/FA-D2c (FANCD2 complemented) and FA-G/FA-Gc cultivated in 3% and 20% of oxygen. Cell cycle studies and cell count showed a general increase of proliferation in pO_2 3%, especially in mutant cell lines, with a marked difference in FA-G/FA-Gc pair. The level of ROS was higher in pO_2 20% and in mutant vs complemented cell lines and was accompanied by the drop in mitochondrial membrane potential and GSH level. The genes involved in DNA repair, hOGG1 and RAD51 and apoptosis resistance related to oxidative stress-Bcl2 were also studied. mRNA level was lower in cells at pO_2 3% for all genes and cell types. FA-Gc and FA-D2c had higher levels of hOGG1 and RAD51 mRNA only at pO_2 20%, whilst bcl-2 mRNA was augmented in complemented cell lines regardless of pO_2. The molecular basis of FA cells sensitivity to oxygen requires additional studies and cell culture conditions, especially oxygen level, could be of crucial importance.

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P-102

N-aceylcisteine and L-arginine reduce endothelial activation and blood pressure

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Patients with type 2 diabetes mellitus (T2DM) present high mortality rate due to cardiovascular disease: reactive oxygen and nitrogen species have been considered involved in the complications occurring in these patients. A decreased availability of nitric oxide (NO) has increasingly gained credit as responsible for endothelial dysfunction and also the presence of some detrimental effects due to the simultaneous presence of oxygen radicals has been postulated. This was the rationale for the use in this study of L-arginine (ARG) and N-acetylcisteine (NAC) administration per os for 6 months in hypertensive T2DM patients. The NAC + ARG treatment caused a reduction of the systolic and diastolic arterial pressure and of the media-intima thickness. Endothelial markers of the atherogenic process activation, such as ICAM-1, VCAM-1 and plasminogen activator inhibitor 1 (PAI-1), significantly decreased. Some markers of inflammation, as the CRP and fibrinogen, and markers of oxidation, such as oxidized LDL and nitrotyrosine, also decreased. These results give prominence to the potential use of oral NAC + ARG treatment for primary and secondary cardiovascular prevention in T2DM patients. However, further clinical studies on a larger scale are needed to support these data.

P-103

Effect of vitamin C administration in the haematological adaptations to an intermittent normobaric hypoxic protocol: Role of free radicals

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Hypoxia induces oxidative stress. In different *in vitro* models it has been suggested that free radicals are involved in the stabilization of the hypoxia-inducible factor 1 (HIF-1). HIF-1 acts as a master regulator of the oxygen homeostasis. Nevertheless, in *in vivo* models, there is little evidence about the role of free radicals in the stabilization of the transcription factor and their effect on the hypoxic-induced haematological adaptations. The major aim of our study was to determine whether normobaric hypoxic protocols induce oxidative stress and whether the administration of antioxidant vitamins modulates the haematological adaptations induced by this type of protocols. Twenty male Wistar rats were randomly divided into four groups: normoxic control ($n = 5$), normoxic treated with vitamin C ($n = 5$), hypoxic control ($n = 5$) and hypoxic treated with vitamin C ($n = 5$). We administered a daily dose of 250 mg/Kg. Reduced (GSH) and oxidized (GSSG) glutathione levels, MDA levels and xanthine oxidase activity were determined in the blood, liver, lung and kidney of our animals. We also determined haemoglobin, reticulocytes (%) and erythropoietin (Epo) plasma levels. Our results show a significant increase in the haemoglobin levels in the hypoxic groups. However, this effect was attenuated with the administrations of vitamin C. All the oxidative stress parameters were also modified in the hypoxic animals and in those treated with vitamin C. In conclusion, free radicals generated during an intermittent hypoxic protocol are involved in the haematological adaptations induced in this type of protocols.

P-104

Nitric oxide down-regulates muscle caveolin-3 levels through S-nitrosylation of myogenin, its transcription factor

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Certain patients suffering from chronic diseases such as AIDS or cancer experience a constant cellular secretion of TNF-α and other pro-inflammatory cytokines that results in a continuous release of nitric oxide (·NO) to the bloodstream. One immediate consequence of the deleterious action of ·NO is weight loss and the progressive destruction of muscular mass in a process known as cachexia. Caveolin-3, a specific marker of muscle cells, becomes down-regulated by the action of ·NO on muscular myotubes. We describe herein that the changes observed in caveolin-3 levels are due to the alteration of the DNA-binding activity of the muscular transcription factor myogenin, by a cGMP-independent mechanism. In the presence of ·NO the binding of transcription factors from cell nuclear extracts of muscular tissues to the E boxes present in the caveolin-3 promoter become substantially reduced. When we purified recombinant myogenin and treated it with ·NO donors we could detect its S-nitrosylation by three independent methods, suggesting that very likely one of the cysteine residues of the molecule, close to the DNA-binding region, is being modified. Given the role of myogenin as a regulatory protein that determines the level of multiple muscle genes expressed during late myogenesis, our results might represent a novel mode of regulation of muscle development under conditions of nitric oxide-mediated toxicity.

P-105

Beer polyphenols and development of atherosclerosis

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It has been demonstrated that moderate consumption of alcoholic beverages reduces cardiovascular mortality probably associated with their content of polyphenols. The apoE is a protein related to the metabolism of the lipoproteins that protects against the atherosclerosis. Thus, the apoE deficient mice have high levels of lipids and cholesterol and develop atherosclerotic lesions. The aim of our study was to daily supply dealcoholized lager and bock beers to apoE deficient mice for 20

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weeks and to observe the possible beneficial effects in the development of the atherosclerosis and the associated mechanisms. Histological lesions of the disease were analysed and the expression of endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) were measured by Western Blotting and reverse transcription PCR. As the internalization of the monocytes to the sub-endothelial space is a crucial step for the development of the disease, we also studied the expression of some adhesion molecules of the endothelium such as P-selectin, V-CAM and I-CAM by immunofluorescence and reverse transcription PCR. The results indicate that the atherosclerotic lesions were reduced by $\sim 50\%$ in treated apoE deficient mice. The expression of the NOS enzymes was maintained. The expression of the P-selectine and ICAM-1 were reduced by 15% whereas VCAM-1 was reduced by 20 and 30% in mice fed lager- and bock-rich diets, respectively. Therefore, it is likely that a reduced internalization of monocytes through the endothelium would be a mechanism to explain the beneficial effect of beer polyphenols at vascular level.

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P-106

Fatty acid synthase down-regulation increases the resistance of Saccharomyces cerevisiae cells to H_2O_2

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Changes in plasma membrane permeability caused by H_2O_2 were recently found to be involved in adaptation to H_2O_2, but the mechanism responsible for this change remains largely unknown. Here this mechanism was addressed and two lines of evidence showed for the first time that fatty acid synthase (Fas) plays a key role during cellular response to H_2O_2: (1) adaptation was associated with a decrease in both Fas expression and activity; (2) more importantly, decreasing Fas activity by 50% through deletion of one of the *FAS* alleles increased the resistance to lethal doses of H_2O_2. The mechanism by which a decrease of Fas expression causes a higher resistance to H_2O_2 was not fully elucidated. However, the *fas1*Δ strain plasma membrane had large increases in the levels of lignoceric acid (C24:0) (40%) and cerotic acid (C26:0) (50%), suggesting that alterations in the plasma membrane composition are involved. Very-long chain fatty acids (VLCFA) through inter-digitation or by modulating formation of lipid rafts may decrease the overall or localized plasma membrane permeability to H_2O_2, respectively, thus conferring a higher resistance to H_2O_2.

P-107

Degradation of hyaluronan by an oxidative system comprising cupric ions and hydrogen peroxide

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The glycosaminoglycan, hyaluronan (HA), has been shown to act as an antioxidant towards reactive oxygen species (ROS), such as hydroxyl radicals (•OH). Three different HA-derived commercial available products (Durolane, Synvisc and Condrox) were depolymerized by hydroxyl radicals. The purpose of this study was to elucidate if the differences in their chemical features lead to different behaviour against •OH. ROS were generated via a Fenton-analogous reaction in which hydrogen peroxide and cupric ions are involved. Change in hyaluronan molecular weight was observed by size-exclusion chromatography. HA degradation percentages were estimated from the retention times observed. It was found that HA degradation by five different concentrations of hydrogen peroxide (between 35–350 mM) did not exert a concentration-dependent manner. However, the results showed differences related to their chemical features in their degradation percentages. The percentages obtained were 13–17% for Durolane, 39–45% for Condrox and 43–45% for Synvisc. These differences may be due to Durolane has NASHA (non-animal stabilized HA) technology remaining NASHA fully biocompatible, whereas Condrox is a high-molecular-weight sodium HA non-cross-linked from rooster comb and Synvisc is a high-molecular-weight hylan cross-linked from rooster comb.

P-108

The effect of exercise on NF-κB related gene expression in skeletal muscle of patients with COPD

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The inflammatory response after exercise is well documented in healthy controls. However, it is unclear whether the inflammatory response in COPD is different. The NF-κB transcription factor plays a key role in the induction of pro-inflammatory gene expression. This study was designed to test whether (1) NF-κB is activated in skeletal muscle tissue in response to exercise, (2) if this activation is reflected in increased transcription of pro-inflammatory cytokines and redox-regulating enzymes and (3) if this activation is related to NF-κB activation in blood. Venous blood samples and biopsies of the vastus lateralis muscle were obtained before, immediately after and 2 h after a 10 min sub-maximal cycle ergometer test (70%) from seven COPD patients and seven healthy age-matched controls. At rest, no differences in NF-κB related gene expression in muscle were observed between COPD and controls but NF-κB activation in blood was lower in COPD ($p < 0.05$). Muscle IL-6 mRNA was upregulated immediately after exercise in COPD and controls ($p < 0.01$) and also 2 h after exercise in controls ($p < 0.05$). Remarkably, immediately after exercise IκBα, IL-1β, TNFα mRNA ($p < 0.05$) were increased in controls, whereas no differences were found in COPD. The antioxidant MnSOD was only upregulated in controls 2 h after exercise ($p < 0.05$), whereas thioredoxin tended to be lower after exercise in COPD. Furthermore, iNOS expression was down-regulated in controls 2 h after exercise ($p < 0.05$). In conclusion, the results indicate a blunted response in NF-κB related pro-inflammatory and antioxidant gene expression in skeletal muscle in response to sub-maximal exercise in COPD compared to controls.

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Melatonin treatment ameliorates aged-related changes in guinea pig liver antioxidant enzymes expression

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It is widely accepted that degenerative changes associated with ageing are related to progressive damage by reactive oxygen and nitrogen species in those situations where the anti-oxidative defence systems fails to eliminate them. In this work we have investigated the oxidative stress in senescent compared to adult guinea pigs and the role of exogenous melatonin in ageing process. For this we have use 4- and 20-month-old female guinea pigs. A group of old animals was treated orally with melatonin (2.5 mg/Kg/day) for 4 weeks, mimicking the circadian rhythm of the naturally secreted hormone. Livers were removed after deep halothane anaesthesia and cervical dislocation and were immediately washed with cold saline solution pH 7.4. The tissue was homogenized and used to determine lipoperoxidation and GSH levels. We found that ageing induces oxidative stress by increasing lipid peroxidation and decreasing GSH levels and these changes were partially recovered by melatonin treatment. To establish whether decreased oxidative stress in senescent animals treated melatonin compare to control senescent animals had a molecular explanation, quantitative real-time PCR was performed to determine mRNA levels for antioxidant genes in liver. mRNA levels for glutathione peroxidase were 5.5- and 3.1-fold higher in adults and in senescents treated melatonin than in control senescents and cytochrome c oxidase were 5- and 3.1-fold higher in adults and in senescents treated melatonin than in control senescents. We conclude that the expression of antioxidant enzymes decreases with age and melatonin can ameliorate this decrease.

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Novel anti-serum recognition of DNA modified with products of lipid peroxidation

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Polyunsaturated fats constitute a significant component of the Western diet and have been linked to an increasing incidence of sporadic colon cancer. It has been proposed that one possible cause may be the degradation of polyunsaturated fats during food processing and cooking, resulting in production of multiple reactive carbonyl species (RCS) that can damage nuclear DNA and proteins, particularly in rapidly dividing cells such as those lining colon crypts. We describe here a novel anti-serum (741) raised against DNA modified with RCS, having an order of reactivity to DNA modified with 4-hydroxy-*trans*-2-nonenal > glyoxal > acrolein > crotonaldehyde > malondialdehyde. Reactivity was also observed against Schiff-base type structures in malondialdehyde-modified low-density lipoprotein. Anti-serum 741 was successfully utilized to demonstrate formation of RCS-DNA in a human colon cell model (undifferentiated CRL-1807 cells) exposed to RCS insult derived from endogenous and exogenous (dietary) lipid peroxidation sources. Further, RCS-modified DNA was detected in the crypt area of colon tissue sections by immunohistochemical analysis, utilizing anti-serum 741. These results highlight the increased susceptibility of colon crypt cells to RCS derived from lipid peroxidation and strongly support the potential involvement of dietary lipid peroxidation products in the development of sporadic colon cancer.

P-111

Changes in NO and lipid peroxidation markers after a maximal treadmill run in swimmers and sedentary young men

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Moderate physical exercise has been accepted as health beneficial. For intense and sustained exercise, such as that performed by competition athletes, some controversy still exists. Redox balance is one of the factors that may contribute to the overall influence of exercise on wellbeing. The purpose of our work was to evaluate the effects of maximal exercise and subjects' training conditions on nitric oxide (NO), a cardioprotective vasodilator, and on oxidative damage (lipoperoxidation) markers. Fifteen high competition male swimmers training 17–23 h/week for at least 5 years, and 16 active men not involved in any regular sport, all between 18–25 years old, performed a continuous graded maximal treadmill run with an individualized protocol. Blood was collected before, immediately after and 2 h after the exercise test. Urine samples were collected before and 2 h after the test. Plasma NO was evaluated by the quantification of plasma nitrate and lipoperoxidation by the thiobarbituric acid reactive substances (TBARS) method in plasma and urine. Training had no significant effect on NO or TBARS basal levels or response to maximal exercise. Both NO and p-TBARS decreased just after the exercise boot and returned to basal levels 2 h later. U-TBARS was decreased 2 h after the exercise test. These results suggest that intense exercise doesn't increase NO bioavailability. However, in these young subjects, oxidative damage seems to be controlled. The decrease in plasma TBARS suggests that exercise stimulates the elimination of lipoperoxidation products which couldn't be explained by increased urinary excretion.

P-112

Oxidative DNA damage induced by potassium bromate in repair proficient and deficient CHO cell lines

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Potassium bromate (KBrO3) is a neurotoxic and possible carcinogen (IARC 2B class) known to be a strong oxidizing agent to which human exposure occurs (e.g. food additive, cosmetic products, byproduct of water ozonation). Its oxidative effect doesn't seem to correlate with the formation of the more common reactive oxygen species. It may be correlated with gluthatione depletion and it is known that, in the presence of glutathione and other thiols, bromate is reduced to bromide in a process involving reactive intermediates, e.g. bromine radicals ($Br^{\bullet}$) or oxides ($BrO^{\bullet}$, $BrO_2^{\bullet}$) generating oxidative DNA lesions via the abstraction of one electron from guanine. Either DNA single strand breaks and/or GC > TA transversions induced by guanine oxidation are dependent on DNA repair efficiency, namely base excision repair in which multistep process XRCC1 protein seem to play a significant role. DNA damaging and oxidative effects of KBrO3 (1–10 mM) were evaluated using Comet assay (1 h exposure) and 8-OHdG quantified by HPLC-ECD (1, 2 and 3 h of exposure) in the XRCC1 deficient Chinese hamster ovary (CHO) cell line EM9 and its parental repair proficient AA8 cell line. Comet assay results show significant dose-response effect and an increased susceptibility of EM9 as compared to AA8 ($p < 0.001$). 8OHdG dose-responses were obtained for both cell lines at all the exposure times assayed ($p < 0.001$). No significant increase in 8OHdG results was found when extending the exposure to KBrO3 from 2 to 3 h. No direct correlation was found between 8OHdG and Comet assay results.

P-113

The differential effect of pO_2 on the proliferation in primary vs 3T3 fibroblasts

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Cultivation of cells is performed under condition that cannot and do not replicate normal physiologic conditions. Oxygen is fundamental for life and its concentration is an important signal for virtually all cellular processes [1,2]. We have compared the effects of two oxygen tensions in cultivation conditions: the atmospheric (pO_2 21%) vs physiological oxygen condition (pO_2 3%) in established fibroblast cell line 3T3 and a rat embryonic fibroblast (REF). Oxidative stress parameters, cell proliferation and the expression of cell cycle related proteins were evaluated. At pO_2 21% 3T3 proliferate more than at pO_2 3% where the cell growth is delayed so is the peak of GSH (24 h and 48 h, respectively). REF grow less and have less GSH at pO_2 21% vs 3%. PO_2 21% favours the proliferation of 3T3 fibroblasts, while the primary fibroblasts grow more at physiological oxygen tension. We believe that an oxygen tension affects differently primary cell cultures compared to established cell lines which were continuously selected against the high, atmospheric, oxygen level. The primary cell culture suffers oxidative stress in the process of isolation and cultivation, so the low, physiological level of oxygen is more adequate for the cultivation of these cells [3].

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P-114

Effects of oestradiol on liver mitochondrial function of ovariectomized and tamoxifen-treated female rats

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Since mitochondria play a central role in both cell life and death, a compelling question is whether 17β-oestradiol (E2) modulates mitochondrial function. Tamoxifen (TAM) has anti-oestrogenic effects in

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the breast tissue and is the standard endocrine treatment for post-menopausal women with breast cancer. However, it is now known that under certain circumstances and in certain tissues, in addition to acting as a competitive inhibitor of endogenous oestrogen, it can also exert oestrogenic agonist properties. In this line, we evaluated the effect of E2 on liver mitochondria isolated from control, ovariectomized (OVX) and TAM-treated ovariectomized (OVX + TAM) rat females. We observed that at basal conditions, ovariectomy and TAM treatment do not induce any statistical alteration in respiratory chain and oxidative phosphorylation system when compared with controls. However, TAM treatment increases the capacity of mitochondria to accumulate Ca^{2+}, delaying the opening of the mitochondrial permeability transition pore (MPTP). The presence of 25 μM E2 impairs respiration and oxidative phosphorylation system, these effects being similar in all groups of animals studied. Concerning the MPTP, we observed that 25 μM E2 increases the probability of MPTP opening, especially in OVX + TAM females. In contrast, E2 protects against lipid peroxidation (TBARS formation and oxygen consumption) in all groups of animals studied. Our results indicate that E2 has, in general, deleterious effects leading to mitochondrial impairment, suggesting that post-menopausal hormonal therapy should be carefully considered. More studies will be useful in clarifying the controversy about the risks vs benefits of E2 supplementation.

P-115

Development of novel mass spectrometry methodology to detect post-translational modifications in oxidative stress and disease

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Oxidative stress is a factor in inflammatory diseases, where immune cells release oxidants that can damage biomolecules, including proteins. The precise nature of oxidative damage to individual proteins depends on the oxidant involved. Chlorination and nitration are examples of oxidative modifications commonly used as markers of damage by myeloperoxidase and nitric oxide-derived oxidants. Although the presence of these modifications can be detected by western blotting, currently no reliable method exists to identify the specific sites damage to individual proteins in complex mixtures such as clinical samples. We have investigated the potential of LCMS and pre-cursor ion scanning methods to provide this information. LC-MS^2 allows separation of peptides and detection of mass changes in oxidized residues, for example by the generation of indicative immonium ions on fragmentation of the peptides (chlorotyrosine immonium ion, m/z 170; nitrotyrosine immonium ion, m/z 181). However, it was found that ions isobaric to these immonium ions exist in unmodified peptides, so this method is prone to false positives. To overcome this, we have optimized the use of a nano-LC/MS^3 mass spectrometry method involving the dissociation of immonium ions to give fragments specific for chlorotyrosine or nitrotyrosine. The method has been validated by comparison with western blotting and has proved able to identify precise protein modifications in complex mixtures of proteins and biological samples subjected to oxidative stress. The application to oxidative modifications of other residues is currently being investigated. This methodology will allow the detection of oxidative post-translational modifications in clinical samples, thus offering potential both for earlier disease diagnosis, monitoring the outcomes of therapy and improved understanding of disease biochemistry.

P-116

Sustained hydrogen peroxide induces iron uptake by transferrin receptor-1 independent of the IRP/IRE network

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Local and systemic inflammatory conditions are characterized by the intracellular deposition of excess iron, which may promote tissue damage via Fenton chemistry. Because the Fenton reactant H_2O_2 is continuously released by inflammatory cells, a tight regulation of iron homeostasis is required. Here, we show that exposure of cultured cells to sustained low levels of H_2O_2 that mimic its release by inflammatory cells leads to upregulation of transferrin receptor 1 (TfR1), the major iron uptake protein. The increase in TfR1 results in increased transferrin-mediated iron uptake and cellular accumulation of the metal. Although iron regulatory protein 1 (IRP1) is transiently activated by H_2O_2, this response is not sufficient to stabilize TfR1 mRNA and to repress the synthesis of the iron storage protein ferritin. The induction of TfR1 is also independent of transcriptional activation via hypoxia inducible factor 1α (HIF-1α) or significant protein stabilization. In contrast, pulse experiments with [^{35}S]-labelled methionine/cysteine revealed an increased rate of TfR1 synthesis in cells exposed to sustained low H_2O_2 levels. Our results suggest a novel mechanism of iron accumulation by sustained H_2O_2, based on the translational activation of TfR1, which could provide an important (patho)physiological link between iron metabolism and inflammation.

P-117

Manganese enhances oxidative damage to cellular and isolated DNA induced by a coffee polyphenol, chlorogenic acid

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Chlorogenic acid is found in many fruits and coffee. The International Agency for Research on Cancer (IARC) has classified coffee consumption and caffeic acid, a major metabolite of chlorogenic acid, as group 2B carcinogens. We investigated the DNA-damaging ability of chlorogenic acid in human cultured cells. Chlorogenic acid and Mn(II) increased the formation of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), an indicator of oxidative damage, in human cultured HL-60 cells, but not in its H_2O_2-resistant clone HP100. To clarify the mechanism of oxidative DNA damage, we performed experiments using ^{32}P-labelled DNA fragments obtained from genes relevant to human cancer. Chlorogenic acid induced DNA damage in the presence of Cu(II) and NADH and the addition of Mn(II) enhanced it. Catalase and bathocuproine inhibited DNA damage, indicating the involvement of H_2O_2 and Cu(I). Piperidine and formamidopyrimidine-DNA glycosylase (Fpg) treatments revealed that chlorogenic acid induced damage to the cytosine and guanine residues of the 5′-ACG-3′ sequence complementary to codon 273, a well-known hotspot of the human *p53* tumour suppressor gene. Mn(II) enhanced chlorogenic acid-induced DNA damage including 8-oxodG formation in the presence of Cu(II) and NADH. UV-visible spectroscopic measurements showed that Mn(II) enhanced autoxidation of chlorogenic acid and NADH consumption. In conclusion, the present study demonstrated that chlorogenic acid could induce oxidative damage to cellular and isolated DNA in the presence of Mn(II), which is included in vegetables, fruits and coffee.

P-118

Oxidative stress and inflammatory markers in advanced coronary artery disease

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The disbalance of redox equilibrium and chronic inflammatory status play a role in pathophysiology of atherosclerosis. The aim was to asses the impact of systemic inflammation, oxidative stress and anti-oxidative status in patients with advanced coronary artery disease. Inflammatory and oxidative stress parameters were determined in group of patients with serious coronary artery disease (at least 50% stenosis of the left main coronary artery or 70% stenosis of other coronary artery according to coronarographic examination (S, $n = 40$) and control group of healthy people (C, $n = 48$). Total amount of free radicals was determined by spectrophotometric assay; malondialdehyde, glutathione, homocysteine and alpha-tocopherol plasma concentrations by HPLC. Plasma levels of hsCRP, $alpha_1$antiproteinase, total cholesterol, triglycerides, HDL-ch, LDL-ch, albumin, fibrinogen were monitored. In patients with coronary stenosis (S) higher level of free radicals (FR) coincided with higher level of hsCRP and lower level of lipid standardized alpha-tocopherol (AT). FR: S: 5.11 ± 0.58 mmol/l vs

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C: 4.60 ± 0.79 mmol/l ($p < 0.01$); hsCRP: S: 4.44 ± 2.78 mg/l vs C: 1.72 ± 1.19 mg/l ($p < 0.01$); AT: S: 2.78 ± 0.45 μM/l vs C: 3.26 ± 0.55 μM/l ($p < 0.05$). In S group significantly higher levels of homocysteine, alpha$_1$antiproteinase, triglycerides, fibrinogen and lower level of albumin were found. In conclusion, increased level of FR in patients was related to the presence of systemic inflammation and lower lipid-standardized alpha-tocopherol, suggesting that abnormalities in both chronic inflammatory status and anti-oxidative substance levels underlie the increased oxidative stress in advanced atherosclerosis, which is further potentiated by homocysteine, fibrinogen and lipid higher levels.

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40% and 80% methionine restriction without changing other dietary components decrease oxidative stress in rat liver

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It is well known that 40% dietary restriction (DR) lowers mitochondrial reactive oxygen species (ROS) generation and oxidative damage and increases maximum longevity. Protein and methionine restriction (MetR) also increase maximum longevity and they also lower mitochondrial ROS generation and oxidative stress. However, previous experiments of MetR were performed only at 80% restriction and substituting dietary methionine for glutamate in the diet. In the present study, Wistar rats were subjected to 40% and 80% MetR during 6–7 weeks without changing other dietary components. It was found that both 40% and 80% MetR decrease mitochondrial ROS generation in rat liver mitochondria without altering mitochondrial oxygen consumption, similarly to what has been previously observed in 40% protein restriction and 40% DR. These decreases in ROS generation were associated to decreases in complex I and III content, which are known to be responsible for mitochondrial ROS generation. In agreement with the decrease in ROS generation, oxidative damage to mitochondrial DNA and five different markers of protein oxidative damage were also decreased by 40% and 80% MetR in liver mitochondria. The decrease in protein lipoxidation occurred together with a decrease in membrane unsaturation, similarly to the changes observed when comparing mammalian species with different longevities. These results support the possibility that MetR intake is responsible for the decrease in mitochondrial ROS generation and oxidative stress and for part of the increase in longevity observed in DR.

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Doxorubicin-induced NF-κB transcriptional repression is dependent on superoxide anions

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Doxorubicin (DOX), an anthracycline antibiotic, is widely used for the treatment of several cancers. However, the clinical efficacy of this drug is limited due to the continuous generation of reactive oxygen species (ROS) via a redox-cycling mechanism during its metabolism. ROS can induce signalling cascades at moderate concentrations, playing an important role as regulatory mediators. We previously demonstrated that in primary cultures of rat hepatocytes DOX induced in a dose- and time-dependent manner NF-κB translocation into the nucleus, delivering a complex that was able to bind to its consensus DNA sequence. In the present report we analysed by RT-PCR the expression of genes known to be under NF-κB control. Surprisingly, the mRNA levels of IκB-α, IκB-β, iNOS, γ-GCS, Bax, p53, Bcl-2, Bcl-xS and Bcl-xL decreased after treating the cells with DOX for 24 h. The addition of MnTBAP (a cell-permeable superoxide dismutase mimetic) restored the mRNA levels of all the studied genes to control values. These data show that DOX-induced activation of the NF-κB signalling pathway renders a transcription repressing complex and suggest a role for ROS, particularly superoxide anions, in the transcriptional repression of NF-κB.

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Effect of beer polyphenols on angiogenesis

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Beer is one of the most commonly consumed alcoholic beverages. Hops used in beer production are an important source of polyphenols, such as xanthohumol (XN), isoxanthohumol (IXN) and 8-prenylnaringenin (8PN). Most XN is converted to IXN during the brewing process. Furthermore, 8PN is formed during drying, storage and extraction of hops. Consumption of a diet rich in polyphenols inversely correlates with development of cancer and atherosclerosis, two frequent disorders in the western world, which present angiogenesis and oxidative stress in common. Given the well-established antioxidant and anti-angiogenic characteristics of polyphenols, the aim of this study was to investigate the effect of XN, IXN and 8PN in the angiogenic process. Human umbilical vein endothelial cells (HUVEC) and human aortic smooth muscle cell (HASMC) cultures were treated with 0.01–20.0 μM XN, IXN and 8PN for 24 h. These three polyphenolic compounds presented anti-angiogenic properties, as observed by their effects on cell viability (MTT assay), apoptosis (TUNEL assay), migration (injury assay) and invasion (double-chamber assay), as well as in capillary-like structures formation in HUVEC cultures alone or in the presence of HASMC on Matrigel. Interestingly, XN seems to strictly target angiogenic vessels, without exerting adverse effects on stable vessels. These results suggest that polyphenols might be useful therapeutic agents against pathological situations where angiogenesis is involved. In addition, these findings emphasize the distinct effects exerted by compounds with similar chemical structures.

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The dual role of catechol oestrogen metabolites in breast cancer: Potent pro-oxidants and aromatase inhibitors

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High levels of endogenous oestrogens are associated with an increased risk of breast cancer. Oestrogen levels increase with the increase in aromatase activity, the cytochrome P450 enzyme responsible for oestrogen formation and decrease by oxidative and conjugative metabolism. Phase I metabolites, the 2- and 4-hydroxy catechol derivatives, can undergo metabolic redox cycling with generation of quinones/semi-quinones and free radicals, electrophilic intermediates that bind covalently to DNA inducing mutations. Although 4-hydroxyestradiol has been shown to be a potent carcinogen both *in vitro* and *in vivo*, several C2-metabolites demonstrate opposite activity. In this work, we have focused on the biochemical evaluation of the main oestradiol and oestrone metabolites as inhibitors of the aromatase enzyme activity. The compounds were tested *in vitro* in an enzymatic assay with aromatase extracted from human term placenta. Full dose-response curves were obtained allowing the determination of the half maximal inhibitory concentration (IC_{50}) and these results were compared with the IC_{50} of a first generation aromatase inhibitor with clinical activity. Aromatase kinetic analysis in the presence and in the absence of the compounds, allowed the evaluation of the type of inhibition and the inhibitory constant, K_i, for the more potent inhibitor tested. Furthermore, a rationale for the molecular interaction with the aromatase binding pocket was proposed based on the Electrostatic Surface Potentials (ESP) derived from *ab initio* quantum chemical methods.

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Mechanisms of PC-12 cell death exposed to nitric oxide and 3,4-dihydroxyphenylacetic acid

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Parkinson's disease is one of the most common neurodegenerative diseases characterized by the selective degeneration of dopaminergic neurons in the *substantia nigra pars compacta.* Although the precise mechanisms underlying the cell death in Parkinson's disease are not clearly established, there are many studies suggesting that an excessive or inappropriate formation of nitric oxide (•NO) (and its derivatives) and an abnormal metabolism of dopamine may modulate cell events that are involved in the ethiology of the disease. In the present study, we establish a pathway for cell death in PC-12 cells, a cell line often used as a neuronal model, involving •NO and 3,4-dihydroxyphenylacetic acid, (DOPAC) a major metabolite of dopamine. Our data shows that DOPAC is not only able to enhance cell death induced by •NO but also modifies the type of programmed cell death induced by the later compound. A remarkable difference is the lack of caspases activation in the case of simultaneous exposure to co-incubation with DOPAC and •NO as compared with the exposure to •NO alone, in which caspases activation is an ordinary event. Preliminary results point to the involvement of AIF as a key mediator in the combined effect of DOPAC and •NO on the cell viability. Also, GSH is involved as a modulator of cell death, as indicated by the following observations: (a) when the cells are co-incubated with DOPAC and •NO, a depletion of GSH content precedes cell death and (b) a pre-incubation of the cells with GSH ethyl ester affords c.a 100% of protection against death of the cells exposed to DOPAC and •NO. The results suggest a mechanism of death involving a complex interaction between •NO and DOPAC in dopaminergic cells that may underlie cell demise associated with Parkinson's disease.

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Metal ion independent antioxidant activity of albumin and its effect on oxidation of LDL

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It is well known that serum albumin acts as an antioxidant by different mechanisms including chelation of metal ions and scavenging of reactive species. However, different methods provide quite different quantitative results. The contribution of several amino acids, e.g. cysteine and methionine, to the antioxidant capacity of albumin has been shown. The *in vivo* relevance of the antioxidant albumin, however, remains unclear. The aim of our study was to investigate the metal chelating independent antioxidant activity of albumin and to demonstrate the effect of albumin on copper independent oxidation of LDL. Depending on the method used albumin shows an antioxidative capacity ranging from essentially zero to a better effect as found for glutathione. The kinetics of the reaction with different reactive species is slow compared to small molecule antioxidants like ascorbic acid. Kinetics is comparable to thiol containing compounds after the start of the reaction and to tyrosine in a later phase. Albumin in physiologically relevant concentrations inhibits oxidation of LDL induced by peroxyl radicals demonstrated by formation of lipid hydroperoxides, epitopes of oxidized LDL, TBARS and electrophoretic mobility. Upon reaction with peroxyl radicals the free thiol group of cysteine-34 is oxidized.

P-125

Antioxidant and vasorelaxant activities of varietal portuguese red wines: The contribution of the anthocyanin content

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The potential biological benefits of wine, in the context of cardiovascular diseases prevention, depend on its polyphenol composition and among these compounds, anthocyanins have raised much interest as red wine anti-atherogenic factors. The aim of this work was to study the anthocyanin composition of three red wines from typical Portuguese grape species, Jaen (JN), Touriga Nacional (TN) and Baga (BG) of the same year grape harvest and to compare their antioxidant activities against human LDL oxidation and their abilities to induce endothelium-dependent relaxation via NO-release, attempting to clarify the contribution of such compounds for these activities. Anthocyanin analysis, by HPLC/DAD, of the wines and the respective isolated anthocyanin fractions, showed similar qualitative profiles but significant quantitative differences. Jaen and Touriga Nacional wines and respective extracts have the higher anthocyanin contents, but in all of them, Mv3glc is the main anthocyanin. Also, all the wines and anthocyanin fractions show strong antioxidant activities against free radical-induced LDL oxidation, as evaluated by conjugated dienes formation. Concerning the vasorelaxant studies, our data show a good correlation between the endothelium-dependent relaxation of pre-contracted rat aortic rings, via NO-release, promoted by wines and fractions and their total anthocyanin concentrations. In conclusion, these compounds, beyond the strong protective role against LDL oxidation, contribute to the wine-induced vascular relaxation, via NO release, and consequently to the beneficial role of regular and moderate red wine consumption in cardiovascular diseases prevention.

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Cytoprotective effect of anthocyanins against peroxynitrite-promoted endothelial cells toxicity in relation to their antioxidant activities

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Anthocyanins are a group of flavonoid pigments widely distributed in fruits, vegetables and red wine. There are several examples of their potential health benefits which include the prevention of cardiovascular diseases, anti-tumourigenic, anti-inflammatory and anti-microbial properties and, more recently, neuroprotective actions. These so called phytonutrients, despite having sugar residues bound to their main structure, are able to pass through the gut wall and enter into blood circulation intact. Thus, the aim of the present work was mainly to compare the protection afforded by four anthocyanins, malvidin- (Mv3glc), cyanidin- (Cy3glc), delphinidin- (Dp3glc) and pelargonidin-3-glucoside (Pg3glc), against peroxynitrite-promoted endothelial cells toxicity and compare such effects with their antioxidant activities. It was also our goal to evaluate how their substitution patterns in the B ring of the aglycon influence their activities. Our data indicate that all of the anthocyanins (up to 50 μM) are non-toxic to bovine aortic endothelial cells in primary cultures (BAEC), according to MTT test. Furthermore, pre-incubation of cells with these compounds protect them from death promoted by peroxynitrite exposure, a relevant oxidant in the context of atherogenesis. Preliminary results indicate that Cy3glc and Dp3glc, both with an ortho-dihydroxyl group in B ring, are the most efficient, while Pg3glc, with a monophenol structure, exhibits the lowest protective action. A similar trend was obtained in relation to their peroxynitrite and peroxyl radical trapping activities, as evaluated by the inhibition of peroxynitrite-mediated dihydrorhodamine oxidation and of LDL oxidation promoted by AAPH-generated peroxyl radicals, respectively, pointing to the relevance of particular structural features to such activities. Although much still remains to be discovered concerning to anthocyanins behaviour *in vivo*, they undoubtedly possess an enormous health-promotion potential, particularly in the context of atherogenesis prevention.

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Hyperglycemia decreases mitochondrial function: The regulatory role of mitochondrial biogenesis

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Increased generation of reactive oxygen species (ROS) is implicated in 'glucose toxicity' in diabetes. However, little is known about the action of glucose on the expression of transcription factors in hepatocytes, especially those involved in mitochondrial DNA (mtDNA) replication and transcription. Since mitochondrial functional capacity is dynamically regulated, we hypothesized that stressful conditions of hyperglycemia induce adaptations in the transcriptional control of cellular energy metabolism, including inhibition of mitochondrial biogenesis and oxidative metabolism. Cell viability, mitochondrial respiration, ROS generation and oxidized proteins were determined in HepG2 cells cultured in the presence of either 5.5 mM (control) or 30 mM glucose (high glucose) for 48 h, 96 h and 7 days. Additionally, mtDNA abundance, plasminogen activator inhibitor-1 (PAI-1), mitochondrial transcription factor A (TFAM) and nuclear respiratory factor-1 (NRF-1) transcripts were evaluated by real time PCR. High glucose induced a progressive increase in ROS generation and accumulation of oxidized proteins, with no changes in cell viability. Increased expression of PAI-1 was observed as early as 96 h of exposure to high glucose. After 7 days in hyperglycemia, HepG2 cells exhibited inhibited uncoupled respiration and decreased MitoTracker Red fluorescence associated with a 25% decrease in mtDNA and 16% decrease in TFAM transcripts. These results indicate that glucose may regulate mtDNA copy number by modulating the transcriptional activity of TFAM in response to hyperglycemia-induced ROS production. The decrease of mtDNA content and inhibition of mitochondrial function may be pathogenic hallmarks in the altered metabolic status associated with diabetes.

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Passive stretching of isolated mature single skeletal muscle fibres. Does it augment the intracellular content of ROS and/or NO?

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Reactive oxygen species (ROS) and nitric oxide (NO) are constantly produced by skeletal muscle and may play an important role in signalling and regulatory pathways. Release of extracellular NO was originally demonstrated in isolated muscles *in vitro* [1] and passive stretching of muscle has been shown to increase NO release from rat skeletal muscle *in vitro* [2]. The aim of the present study was to determine whether application of passive stretching to single mature skeletal muscle fibres augments the generation of intracellular ROS and/or NO. Muscle fibres were isolated from the *Flexor Digitorus Brevis* of mice and attached to a flexible silicone membrane which had been previously coated with a collagen Matrigel™ matrix. In order to detect the intracellular generation of ROS and/or NO, fibres were loaded with different fluorophore probes: DCFH (general detector of ROS), hydroethidine (detector of superoxide) and DAF-FM (detector of NO). The passive stretching protocol was applied to fibres using the FX-4000™ Flexercell® system for a fixed period of the experiment time course. Using fluorescence microscopy, fluorescence emission from fibres was photographed at different time points and quantified by imaging analysis. The results indicate that the passive stretching protocol does not induce significant changes in intracellular fluorescence from single mature skeletal muscle fibres which have been previously loaded with each of the fluorophore probes. However, the emission of fluorescence was significant when the fibres were exposed to different positive controls, indicating that the technique was sensitive enough to detect changes of the intracellular ROS and/or NO. In conclusion, passive stretching applied to single mature skeletal muscle fibres does not induce a significant increase in the generation of intracellular ROS or NO.

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Anti-inflammatory effects of phenylpropanoids on human keratinocytes

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Oxidative stress plays a relevant role in the pathogenesis of inflammatory disorders, including chronic diseases of the skin. Notably, distinct classes of antioxidant principles may also possess anti-inflammatory activity. We evaluated the effect of two novel natural polyphenolic antioxidants with phenylpropanoid structure, identified as Teupolioside and Verbascoside, on the inflammatory activation of keratinocytes. Escalating concentration of each of the two pure principles were added to keratinocyte cultures 1 h before administration of TNF-α plus IFN-β and this incubation was then maintained for a further 24 h. The results showed that these substances inhibited keratinocyte release of crucial pro-inflammatory mediators, including the cytokine GM-CSF and the chemokines CXCL10/IP-10, CCL2/MCP-1 and CXCL8/IL-8 in a dose-dependent fashion and with a potency similar to that displayed by the corticosteroids hydrocortisone and triamcinolone in the same experimental model. We found that Teupolioside and Verbascoside dramatically impaired NFκB binding activity in cytokine-stimulated cultures and concomitantly abrogated ERK1/2 phosphorylation and activity. These results suggest that these anti-oxidant principles could be of potential interest in the treatment of chronic inflammatory skin disorders.

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Plant polyphenols against UVC-induced necrosis and apoptosis of keratinocytes

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UV exposure leads to the impairment of antioxidant protection and oxidative damage of the skin. Certain polyphenols are synthesized in the plants in order to protect them against UV-induced oxidative damage. The same plant-derived polyphenols are widely used as UV-screens for topical application in humans. The study was designed to evaluate the effects of phenylpropanoid glycosides (verbascoside) and flavonoids (rutin and quercetin) towards UVC-induced ROS-dependent necrosis and apoptosis in HaCaT human keratinocytes. Two exposure protocols, 1 and 10 min long, were applied. Cell death was assessed by the enzyme leakage analysis (necrosis) and the differential permeability for the DNA fluorescent dyes ethidium bromide and acridine orange (apoptosis). Our data revealed that a 10 min long UVC irradiation resulted in a rapid (within 1 h) cell death by necrosis while a 1 min long irradiation led to blocking of keratinocyte proliferation and triggered apoptosis. All plant polyphenols studied were effective against the loss of membrane integrity in doses consistent with their antioxidant action. At the same time, all three compounds did not affect significantly the UVC-induced proliferation arrest and apoptosis. Contrary, the data obtained suggested that certain plant polyphenols could switch the UVC–induced cell death from necrosis to apoptosis.

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The time-dependent effect of L-NAME on nitric oxide synthase isoform expressions in the heart and brain

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The goal of our study was to analyse a time course of L-NAME effects on eNOS, nNOS and iNOS protein expressions, NOS activity, membrane oxidative damage and blood pressure (BP), respectively. Adult 12-week-old male Wistar rats were divided into three groups: control, L-NAME (40 mg/kg/day) for 4 weeks and L-NAME (40 mg/kg/day) for 7 weeks. Both 4- and 7-week-L-NAME treatments increased BP in comparison with controls. After 4 weeks of L-NAME treatment eNOS expression in the heart increased significantly and this increase was amplyfied after 7 weeks of treatment. On the other hand, eNOS expression in the brain remained unchanged after

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4-week-L-NAME treatment and prolonging the treatment led to significant decrease of eNOS expression in this tissue. There were no changes in protein expressions of other isoforms. NOS activity was decreased after 4 weeks of L-NAME treatment in both tissues. However, prolonging the treatment to 7 weeks increased NOS activity in the heart while NOS activity in the brain was decreased more significantly. Oxidative damage was detected in both tissues after 4-week-L-NAME treatment. Prolonging the treatment amplyfied the damage in the brain only. In conclusion, increased expression of eNOS may be responsible for increased NOS activity and reduced oxidative damage in the heart after 7-week-L-NAME treatment. Decreased expression of eNOS led, however, to more significant decrease of NOS activity and oxidative damage in the brain. Since BP increase persisted after 7 weeks of L-NAME treatment, we hypothetized that central regulation of BP is predominant in L-NAME-induced hypertension.

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The mechanism of Apocynin-induced prevention of borderline and spontaneous hypertension

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We aimed to study the preventive effect of NADPH oxidase inhibitor, apocynin, on blood pressure (BP) increase in borderline hypertensive (BHR) and spontaneously hypertensive rats (SHR). Young 6-week-old male BHR (offspring of SHR dams and Wistar Kyoto sires), SHR and Wistar Kyoto (WKY) were treated with apocynin in the dose of 30 mg/kg/day for 6 weeks. Nitric oxide synthase (NOS) activity was determined in the aorta, left ventricle and kidney. Concentration of conjugated dienes (CD) and cGMP and expression of nuclear factor NF-kappaβ and NADPH oxidase sub-units were detected in the left ventricle and kidney. BP of WKY, BHR and SHR was 119 ± 2, 144 ± 3 and 189 ± 2 mmHg, respectively, at the end of experiment. NOS activity was increased in the aorta, left ventricle and kidney of both hypertensive groups comparing to normotensive WKY. Apocynin (A) significantly decreased BP rise in both hypertensive groups (131 ± 2 mmHg in BHR + A and 144 ± 2 mmHg in SHR + A) and lowered CD concentration in the kidney. Moreover, apocynin decreased expression of NF-kappaβ in the left ventricle of both hypertensive groups. Concurrently, expression of NADPH oxidase sub-units was decreased in SHR treated with apocynin. Despite the disability of apocynin to influence NOS activity, it was able to increase cGMP concentration in the left ventricle and kidney with more pronounced effect in SHR. In conclusion, apocynin without effecting NOS activity decreased level of reactive oxygen species leading to the increased concentration of cGMP with preventing effect on blood pressure rise in both borderline and spontaneously hypertensive rats.

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Effect of fermentation after harvest on the total antioxidant activity of *Tetragonisca angustula* honey

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After harvest, stingless bee tropical honey produce froth due to the higher water content than *Apis mellifera* honey. In this work, the antioxidant capacity of fresh *Tetragonisca angustula* honey with 26.2 g water/100 g honey was stored at two temperatures, at 4°C and 30°C, was measured every 5 days up to a month. Artificial and pasteurized honeys also kept at 4°C and 30°C were the controls. The antioxidant indicators were the inhibition percentage of superoxide anion ($O^{2-\bullet}$) and hydroxyl radical ($OH^{\bullet}$) and the benzoate degradation known as antioxidant activity (AOA). Concentrations of proteins, total sugars and ethanol were also monitored for 1 month. A gradual increase in ethanol only occurred in the honey kept at 30°C (8.7 to 29.3 mg/kg), besides a total sugar decrease (81.5 to 31.7 g/100 g honey), indicating honey fermentation. Protein concentration showed a low decrease (207 to 197 mg/100 g honey), while the antioxidant capacity indicators increased (superoxide anion, 69.1 to 94.8%; hydroxyl radical, 62.2 to 90.5%; AOA, 0.58 to 0.89 mM) along with the fermentative process. Fermentation increased the antioxidant bioactivity of *T. angustula* honey. This fact could explain the reputed medicinal properties of stingless bee honey, dating back to the Mayas, by decreasing the oxidative damage to lipids, proteins, carbohydrates and nucleic acids caused by reactive oxygen species (ROS).

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Effects of melatonin, silymarin and ursodeoxycholic acid on maternal cholestasis-induced oxidative stress in the rat foetal liver–placenta–maternal liver trio

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Maternal cholestasis can induce placental damage and may be lethal for the foetus. We have investigated the response of some components involved in the cellular antioxidant defense system to the hypercholanemia and hyperbilirubinemia associated to maternal cholestasis and the potential protective effect of melatonin, silymarin and ursodeoxycholic acid (UDCA) on maternal liver, foetal liver and placenta. Complete biliary obstruction was performed in rats on day 14 of pregnancy. During the following week, some animals received daily intragastric administration of melatonin, silymarin or UDCA. Human liver (hepatoblastoma HepG2) and trophoblast (choriocarcinoma JAr) cell lines incubated with taurocholic acid (TCA), UDCA or bilirubin were used as *in vitro* models. Melatonin, silymarin and UDCA protected against oxidative stress and apoptosis activation induced by maternal cholestasis in the three organs assayed. Maternal cholestasis stimulated the expression of the genes encoding for the biliverdine-IXα reductase and the sodium-dependent vitamin C transport proteins SVCT1 and SVCT2. Melatonin and UDCA, but not silymarin, upregulated the expression of these genes. In HepG2 and JAr cells, bilirubin increased biliverdine-IXα reductase, SVCT1 and SVCT2 mRNA levels. TCA and UDCA induced SVCT2 and biliverdine-IXα reductase mRNA expression in both cell types and that of SVCT1 only in JAr cell line. These results suggest that bile acids and bilirubin accumulation during maternal cholestasis stimulates a response of antioxidant systems in maternal liver, foetal liver and placenta. Melatonin, silymarin and UDCA afford a direct protective antioxidant activity whereas only melatonin and UDCA induce the expression of some components of the antioxidant defense.

P-135

Effects, following ingestion of Savory (*Satureja montana* L.) and Raspberry (*Rubus idaeus* L.) water infusions, upon mice liver oxidative status

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Several plants of traditional use may possess potential of therapeutic applications. The aim of the present research effort was to assess the biosafety and bioactivity (in terms of antioxidant potential) of water infusions of Savory (*Satureja montana* L.) and Raspberry (*Rubus idaeus* L.). Replacement of water by plant aqueous infusions in the diet of mice for 14 days did not affect their body weight or food consumption. Damages of lipids, proteins and DNA were assessed as oxidative status biomarkers. The levels of glutathione (reduced and oxidized forms), as well as the activity of glutathione reductase, glutathione S-transferase, glutathione peroxidase, catalase and superoxide dismutase in the liver have also been determined. Both plant infusions showed a significant protection against lipid oxidation and no significant effect on protein oxidation. As to DNA damage, it has been significantly reduced by Raspberry infusion. Levels of liver glutathione were not significantly

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changed except for the Savory group—for which there was a significant decrease in reduced glutathione, without changes in the activity of the enzymes involved in glutathione metabolism. Concerning catalase and superoxide dismutase, their activity was significantly decreased in the Savory group and exhibited a decreasing tendency in the Raspberry group, in comparison to controls. In conclusion, this study indicated that compounds present in Savory and Raspberry infusions have the capacity to alter the liver oxidative status in the mouse.

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Detection of superoxide radical in living cells by EPR spin-trapping: A step towards *in vivo* EPR imaging

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Reactive oxygen species (ROS) are recognized as regulators of several cell functions, such as growth and differentiation. Electron paramagnetic resonance (EPR) spectroscopy combined with spin-trapping is the method of choice to detect radical species like superoxide or $NO^{\bullet}$ in complex biological systems. Setting up the optimum conditions for detection in intact cells is a first step towards the implementation of *in vivo* EPR imaging, which is available for small animals at UMR8601. Here we present an application of this technique to the detection of superoxide produced by enzymes from the NADPH-oxidase (Nox) family, using a nitrone spin-trap, 5-*tert*-butyloxycarbonyl 5-methyl-1-pyrroline *N*-oxide (BMPO). First, we studied particulate fractions from HEK293 cells expressing Duox2, a glycoflavoprotein involved in thyroid hormone biosynthesis and acting as a H_2O_2 generator functionally associated with thyroperoxidase. Results suggested that Duox2 generate $O_2^{\bullet -}$ that can dismutate into H_2O_2 prior to its release from the protein. Recently, we performed the detection in intact HEK293 cells stably transfected with human Nox5 or with human or porcine Duox2 and investigated the calcium dependence of the superoxide production. We will then evaluate the role of the degree of maturation and glycosylation of Duox2 on the production of H_2O_2 vs superoxide using mutants. Other collaborations for the detection of $O_2^{\bullet -}$, $NO^{\bullet}$ and O_2 in other cell types such as fibroblasts (F. Mechta-Grigoriou, Institut Curie) and chondrocytes (M.-T. Corvol, J.-F. Savouret, Université Paris Descartes), and later on in mice are planned.

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High content bioimaging assays for oxidative stress assessment in primary cultures of hepatocytes

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Analysis of *in vitro* hepatotoxicity is needed to predict drug adverse reactions and to assess the toxic risk of chemical compounds. Such studies are complicated by the difficulty of maintaining some hepatocyte functions in culture and by the functional heterogeneity of liver cells. Furthermore, the need for large number of hepatotoxicity tests in some toxicological settings favours miniaturized and automatized approaches. High-content assays (HCA) based on single-cell, with multiparametric fluorescence measurements of heterogeneous systems, are new alternatives for analysis of cytotoxicity *in vitro*. We present here the preliminary results of our own battery of HCA for oxidative stress assessment, suitable for studies of *in vitro* hepatotoxicity. The functional assays were performed on rat hepatocytes isolated by collagenase perfusion and seeded at low density on optically clear, black-wall 96-well plates layered with collagen. Appropriate dyes were used to assess superoxide, peroxides, glutathione and the oxidized DNA base 8-oxoguanine, in cells treated with vehicle or positive control compounds. All assays have been performed in the automated epifluorescence microscope In Cell Analyser 1000 (GE Healthcare) and the software In Cell Developer Toolbox (GE Healthcare) was used for cell segmentation and data calculation. We conclude that HCA correlate functional responses to morphological and structural parameters in toxicity studies. In addition they reveal hepatocyte heterogeneity and may provide tools to address this phenomenon. Moreover, the 96-well format of HCA enhances data generation while lowering the number of cells per experiment.

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Redoxomics approaches to investigate protein nitration in plasma and dialysis fluids of uremia and dialysis patients

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Different proteomics approaches are used to investigate biomarkers of inflammation and oxidative/nitrosative stress (also known as 'redoxomics'). In this study we applied the classical procedure proposed in redoxomics studies for the analysis of protein nitration in whole plasma and dialysis fluids from patients on regular haemodialysis (HD) therapy [1]. This consists of two-dimension electrophoretic separation, preliminary immunoblotting identification, isolation, digestion and mass spectrometry identity confirmation of proteins that express a specific post-translational modification. Main nitrated proteins preliminarily identified in uremic plasma by immunoblotting and then confirmed by mass spec analysis included albumin, transferrin, complement factor B, IgG1 heavy chain, IgG chain C region, alpha1-antitrypsin and Zn-alpha2-glycoprotein. However, the performance of such analysis was observed to be heavily influenced by the specificity of commercially available antibodies used in the immunoblotting step. To overcome this limit, we propose to adopt a procedure recently proposed by some of us [2], which is based on the mass spec identification of *o*-nitrotyrosine residues in the tryptic fragments by reduction to the corresponding amino-Tyr derivatives and chemoselective labelling with DNS-Cl (dansyl-chloride) to form an *o*-dansylamino-Tyr residue. The redoxomics procedure can be further improved with the elimination of the immunoblotting step and with the introduction of a preliminary step of anti-*o*-nitrotyrosine immunoaffinity chromatography. This improved procedure is expected to be used for an extensive characterization of NOx-derived proteinaceous solutes in HD patients and to assess the performance of various methods and dialysers used in HD therapy.

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Increased circulating myeloperoxidase in women with history of preeclampsia

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Several epidemiological studies have described an association between a history of preeclampsia and increased risk of maternal cardiovascular disease (CVD). This hypertensive disorder shares several features with atherosclerosis, namely the involvement of oxidative stress and endothelial dysfunction. Myeloperoxidase (MPO), a microbicidal haemoprotein normally released from activated monocytes and neutrophils, is an emerging biomarker to assess cardiovascular risk. Since MPO contributes to atherosclerosis through excessive ROS production via the MPO/H_2O_2/Cl oxidation system and its circulating values are

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increased in preeclampsia, we measured MPO circulating levels among women with history of preeclampsia to determine if MPO concentration is still increased 3 and 6 years post-partum. This study includes 33 women with history of preeclampsia between 3–6 years post-partum and 25 matched women with no history of pregnancy complications. In both groups, MPO plasma concentration was measured using a highly sensitive enzyme-linked immunosorbent assay and results were analysed using SPSS®. Plasma MPO concentration is significantly increased ($p = 0.010$) in women with history of preeclampsia compared with women with history of uncomplicated pregnancy (87.3 ± 37.8 vs 62.6 ± 33.0 ng/mL). In conclusion, increased myeloperoxidase plasma levels in women with history of preeclampsia with the subsequent increase ability to produce ROS can be associated with the higher risk of developing cardiovascular diseases. Therefore, it can be seen as a potential molecular target for therapeutic interventions in inflammatory vascular diseases.

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Role of peroxynitrite in endothelial damage mediated by Cyclosporine A: A case for MnSOD

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Whereas Cyclosporine A (CsA) is an effective therapy for immunosuppression, its use encompasses serious side effects that have been associated with oxidative and nitrosative stress. One of the known agents that produces nitroxidative damage is peroxynitrite, the product of the combination of NO with superoxide anion. In this study we report the intracellular formation of both peroxynitrite and 3-nitrotyrosine in cultured bovine aortic endothelial cells (BAEC) and murine aortas, when exposed to CsA. This effect was associated with increased cellular toxicity, which was prevented by N-acetylcysteine. An increase in nitrated MnSOD was detected in BAEC treated with CsA and the peroxynitrite donor SIN-1 and recapitulated in recombinant MnSOD, exposed to the conditioned media from BAEC. We also report the mitochondrial localization of superoxide anion generated by CsA, as detected with the Mitosox red mitochondrial superoxide indicator. Studies using MS-ESI approaches have assigned nitration to Tyr 34 as one of the target residues. We propose that nitration of MnSOD by CsA provides a biochemical mechanism to explain the cellular toxicity of this immunosuppresant, as well as a potential therapeutic target for specific pharmacological or biochemical interventions.

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Oxidative degradation of perlecan, a key matrix proteoglycan, by myeloperoxidase-derived oxidants and its consequences

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The highly basic heme enzymes myeloperoxidase and eosinophil peroxidase utilise H_2O_2 to oxidize halide anions (Cl^-/Br^-) to HOCl and HOBr. Extracellular damage by HOCl/HOBr, which has been detected in human inflammatory lesions using monoclonal antibodies (e.g. mAb 2D10G9), may be localized to the polyanionic glycosaminoglycan components of matrix (proteoglycans and hyaluronan) due to binding of MPO and EPO. We have shown previously that HOCl and HOBr modify and fragment glycosaminoglycans via the formation of *N*-halo derivatives. In our current studies, we have examined the effects of HOCl and HOBr on the heparan sulphate proteoglycan perlecan. Perlecan is a major component of vascular matrix and is a key regulator of vascular cell behaviour and basement membrane function. Perlecan was isolated from human coronary artery endothelial cells (HCAECs) and exposed to varying excesses of HOCl and HOBr. ELISA and immunoblotting studies showed a dose-dependent loss in recognition of the core protein epitopes (domains I, III and V) and the generation of modified protein structures (protein carbonyls and material recognized by mAb 2D10G9). No loss in recognition of heparan sulphate was observed. Extensive protein cross-linking and fragmentation were observed by PAGE. Consistent with these data indicating preferential attack on the core protein, oxidation of perlecan inhibited (core protein-dependent) adhesion of HCAECs but not (heparan sulphate-dependent) binding of basic fibroblast growth factor (FGF-2). Degradation of extracellular matrix by HOCl/HOBr is implicated in pathological tissue changes associated with a number of inflammatory diseases and modification of perlecan may be a key event in these processes.

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Evaluation of spin traps towards the detection of biomolecules free radicals by mass spectrometry

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Free radicals are by-products of aerobic processes that, due to their short-lives, are very reactive species towards biomolecules. These reactions induce modifications to the structure of biomolecules leading to changes in biological functions. The detection of free radicals is usually achieved by stabilizing the free radical with spin traps, usually DMPO, and their detection performed by electron spin resonance (EPR) or mass spectrometry (MS). In this study, four different spin traps, namely two cyclic hydroxylamines (CPH and TMTH), one cyclic nitrone (DEPMPO) and one imidazole (MCPIO) were evaluated towards the detection of hydroxyl, peptide and phospholipid free radicals using mass spectrometry to their detection. Stock solutions of the different spin traps were prepared (5 mM) and diluted by a factor of 10^5 before injection into the mass spectrometer (Q-TOF 2). Upon injection, the spin traps were observed in the mass spectra as $[M+H]^+$ ions. $[M+H]^+$ ions of the cyclic hydroxylamines (CPH and TMTH) showed higher signal intensity followed by the cyclic nitrone (DEPMPO) and the imidazole (MCPIO), when compared to the DMPO spin trap and that can be due to better ionization efficiencies. However, spin trap adducts of hydroxyl radical, $[trap\text{-}OH+H]^+$ was more abundant for DEPMPO and absent for the CPH and TMTH spin traps. In the case of peptide and phospholipid free radicals, the $[trap\text{-}bio+H]^+$ ion was observed for all spin traps although for DEPMPO a higher intensity was observed when compared to the remaining spin traps and to DMPO, suggesting higher trapping efficiency.

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Identification of free radicals of glycerophosphatidylcholines containing ω-6 fatty acids using spin trapping coupled with tandem mass spectrometry

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Oxidative damage to biomolecules triggered by free radicals is an increasing topic of research due to the growing evidence of these reactive species in the pathogenesis of age-related diseases. Metal-catalysed radical oxidation of diacyl-glycerophosphatidylcholines (GPC) bearing one chain of ω-6 acyl polyunsaturated fatty acids (PAPC-palmitoyl-arachidonoyl and PLPC-palmitoyl-lineloyl) was studied. Free radical oxidation products were trapped by spin trapping with 5,5-dimethyl-1-pyrrolidine-N-oxide (DMPO) and identified by Electrospray Mass Spectrometry (ES-MS). The spin adducts of oxidized GPC containing one and two oxygen atoms and one and two DMPO molecules were observed as doubly charged ions. Structural characterization by tandem mass spectrometry (MS/MS) of these ions revealed product ions corresponding to loss of the acyl chains (*sn*-1-palmitoyl and *sn*-2-oxidized spin adduct of lineloyl or arachidonoyl), loss of the spin trap (DMPO) and product ions attributed to oxidized *sn*-2 fatty acid spin adduct (lineloyl and arachidonoyl). Product ions formed by homolytic cleavages near the spin trap together with product ions resulting from 1,4 hydrogen elimination cleavages, involving the hydroxy group in the *sn*-2 fatty acid spin adduct, allowed one to infer the nature and position of the radical formed during oxidation. Altogether, the presence of GPC hydroxy-alkyl/DMPO and hydroxy-alkoxyl/DMPO spin adducts was proposed.

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P-144

Role of liposomes as analytical tools to evaluate the antioxidant profile of pharmaceutical drugs

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Non-steroidal anti-inflammatory drugs (NSAID) from the oxicam family (tenoxicam, lornoxicam, piroxicam and meloxicam) are nowadays rather frequently prescribed because they specifically inhibit the 2-isoform of COX, which could imply a lower incidence of undesirable side-effects. Reactive oxygen species (ROS) play an important role in several pathophysiological processes and are considered mediators of inflammation *in vivo*. It is therefore of interest to more precisely assess the anti-oxidant properties of oxicams which could act in addition to the inhibition of COX. In this study liposomes were used as membrane mimetic systems to estimate the antioxidant properties of oxicams and establish a relationship between the interactions of the drugs with the membrane and their consequent antioxidant activity. Different experiments were performed covering the study of the protective effect of oxicams in lipid peroxidation induced by the peroxyl radical ($ROO^{\bullet}$) derived from 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) and using two fluorescence probes with distinct lipophilic properties. Lipid peroxidation using the hydrophilic probe fluorescein was evaluated in lipid and aqueous media. Lipid systems labelled with the fluorescent probe diphenylhexatriene propionic acid (DPH-PA) were used to assess the effects of the drugs on membrane peroxidation simultaneously by fluorescence intensity decay and changes in membrane fluidity by steady-state anisotropy measurements. Results obtained showed that membrane lipoperoxidation is not only related to the scavenging characteristics of the pharmaceutical drugs studied, but also to their ability to interact with the lipid bilayers and consequently liposomes provide additional information to that obtained currently from assays performed in aqueous buffer media.

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Pinoline prevents changes in the membrane fluidity due to lipid peroxidation in cell membranes from hepatocytes

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Structural changes in cell membranes produced during lipid peroxidation disrupt molecular motion in the membrane. Recently, the antioxidant ability of pinoline has been proposed. We report, using cell membranes isolated from rat liver, the effect of pinoline (0.01–0.4 mM) on membrane fluidity associated with lipid and protein oxidation. Moreover we tested the effect of pinoline on fluidity in the absence of oxidation. Lipid and protein oxidation in cell membranes were induced by addition of $FeCl_3$ (0.1 mM) and ascorbic acid (0.1 mM). Membrane fluidity was measured using fluorescence polarization. Malonaldehyde (MDA) + 4-hydroxyalkenals (4-HDA) concentrations and carbonyl contents were estimated as indices of lipid and protein oxidation, respectively. Pinoline (0.01–0.1 mM) inhibited membrane rigidity in a manner parallel to its preventive effect on lipid and protein oxidation. At a concentration of 0.2–0.4 mM, pinoline increased cell membrane fluidity. When pinoline was tested without induced oxidative damage, 0.2–0.4 mm pinoline maintained the same level of fluidity as that recorded after induced lipid peroxidation. The data reported here may become significant in understanding the role of pineal metabolites related to their protective effect in preventing oxidative damage even at physiological concentrations.

P-146

GRX1 enhances wound repair of alveolar epithelial type II cells *in vitro*

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Recent evidence indicates that oxidants play an important role in cell motility and mobility, at least in part through their influence on the dynamics of the cytoskeleton. In the current study, we demonstrate that protein S-glutathionylation, a redox-dependent post-translational modification, is predominant at the leading edge of a wound in a culture of alveolar epithelial type II cells (A549), from immediately after wounding until closure of the wound. In order to investigate the role of S-glutathionylation in the wound healing process, wounds were treated with 100 or 500 ng/ml recombinant human glutaredoxin 1 (GRX1). This was found to increase wound closure, as did over-expression of GRX1. GRX1 had no effect on cell proliferation, but using Boyden chamber type assays, migration as well as invasion through matrigel were found to be enhanced after GRX1 stimulation. GRX1 furthermore attenuated intracellular levels of S-glutathionylated proteins, including at the edge of the wound. Lastly, actin polymerization was enhanced by GRX1 treatment concomitant with changes in its S-glutathionylation status. Taken together, this study suggests that S-glutathionylation at the edge of a wound in A549 cells in culture is inhibitory to wound closure. Furthermore, GRX1 treatment attenuates intracellular S-glutathionylation and enhances cell motility.

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Oxidative stress in striatal cells expressing mutant huntingtin and in Huntington's disease cybrids

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Huntington's disease (HD) is a neurodegenerative disorder that selectively affects striatal neurons. It is caused by a polyglutamine repeat expansion in the huntingtin protein. Mitochondrial dysfunction has been associated with the generation of reactive oxygen species (ROS), although their involvement in HD is still controversial. Thus, the aim of this work was to elucidate the occurrence of oxidative stress in HD. Striatal cell lines derived from HD knock-in mice, expressing normal (Q7) or mutant (Q111) huntingtin, and cybrid cell lines obtained from the fusion of HD patients (HD cybrids) or control (control cybrids) platelets with mitochondrial DNA-depleted human teratocarcinoma cells were used. Q111 cells revealed increased peroxide formation when exposed to staurosporine, a classic apoptotic inducer, or hydrogen peroxide, when compared to Q7 cells. Upon exposure to rotenone or antimycin A (mitochondrial complex I and III inhibitors, respectively) a significant increase in superoxide anion formation was observed in Q111 cells. Moreover, HD cybrids showed an apparent increase in superoxide anion generation upon exposure to 3-nitropropionic acid, an irreversible inhibitor of complex II. A reduction in superoxide dismutase 2, glutathione peroxidase and glutathione reductase activities was also observed in Q111 cells, after exposure to stress inducers, contributing to the increased levels of endogenous ROS. These results indicate a higher susceptibility of Q111 striatal cells and HD cybrids to oxidative stress, which may underlie the selective cell death in this neurodegenerative disorder.

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P-148

HOCl produced by human neutrophils scavenged by flavonoids

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Hypochlorous acid (HOCl) is a powerful oxidant produced by stimulated neutrophils. This reactive oxygen specie (ROS) generated by reaction of H_2O_2 with Cl^-, catalysed by myeloperoxidase (MPO), has long been recognized to play an important role in the inflammatory process. Inflammation is virtually present in all known diseases, from

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infectious diseases to autoimmune diseases. Therefore, the elimination of HOCl produced in excess may constitute a new therapeutic strategy for those pathologies. The main objective of this work was to evaluate the antioxidant activity of flavonoids (myricetin, quercetin, morin, kaempferol, rutin, luteolin and taxifolin) towards the scaveging of HOCl generated by different systems: HOCl chemically produced; HOCl generated by the MPO/H_2O_2/Cl^- system and finally by human neutrophils. For each flavonoid different IC_{50} values were obtained, when different systems for HOCl generation and detection were used. Although these IC_{50} values are different, the hierarchy for their antioxidant activities is maintained. Quercetin, morin and myricetin display high similar antioxidant activities, luteolin is clearly the less efficient antioxidant and kaempferol, taxifolin and rutin present an intermediate behaviour toward HOCl scavenging. A structure-activity relationship was established with these results. The inhibitory effect of flavonoids on HOCl generation by human neutrophils was also followed inside the cells, by flow cytometry. These assays showed the HOCl generation in real time using a specific probe, the 3-aminophenyfluorescein (APF), as well as cell morphologic changes occurred during stimulation. Furthermore, the structures of chlorinated flavonoids were identified by mass spectrometry.

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Ischemic pre-conditioning prevents I/R injury in fatty liver by increasing mitochondrial tolerance: The key role of mitochondrial ATPase and calcium homeostasis

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The increased susceptibility of steatotic livers to ischemic injury is associated with impairment of cellular energy metabolism. Pre-conditioning has been shown to protect fatty livers from necrosis, possibly through preservation of ATP content. Mitochondria being the main ATP production source for the cell, we aimed to evaluate if ischemic pre-conditioning of fatty livers prevents the impairment in mitochondrial function. Lean and steatotic animals were subjected to 90 min of hepatic warm ischemia and 12 h of reperfusion. Ischemic pre-conditioning effect was tested in fatty livers. After reperfusion, serum AST and ALT levels were measured. Mitochondrial membrane potential, mitochondrial respiration and susceptibility to calcium-induced permeability transition were evaluated, as well as ATPase activity and adenine nucleotides. Pre-conditioning of fatty livers decreased serum AST and ALT levels. Fatty animals subjected to ischemia/reperfusion exhibited decreased mitochondrial membrane potential and a delay in the repolarization after a phosphorylative cycle, associated with increased state 4 respiration. Ischemic pre-conditioning was effective in preventing the impairment of mitochondrial function in fatty livers, also preserving ATPase activity, ATP/ADP ratio as well as calcium homeostasis. Ischemic pre-conditioning of fatty livers preserves mitochondrial function, probably by preventing damage to F_1F_o-ATPsynthase and maintenance of calcium homeostasis.

P-150

Peroxynitrite detoxification by *Leishmania infantum* tryparedoxin peroxidases: Implications for parasite infectivity

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To give rise to a productive infection, *L. infantum* the agent of visceral leishmaniasis might have to deal with ROS/RNS produced by their mammalian hosts. Indeed, parasite phagocytosis has been referred to signal for the assemblage of the NADPH oxidase on the macrophage membrane and, consequently, to induce $O_2^{\bullet -}$ production. On the other hand, macrophage activation by Th1 cytokines such as IFN-γ is linked to induction of iNOS and, therefore, associated with $^{\bullet}NO$ production. A concomitant production of $O_2^{\bullet -}$ and $^{\bullet}NO$ leads to formation of peroxynitrite, a strong oxidant and a cytotoxic molecule. *In vitro*, all these molecules are lethal to parasites and we are studying how the parasite detoxifies them. *Leishmania* possess ROS/RNS detoxification cascades dependent on the unique thiol trypanothione, which involve peroxiredoxins designated as tryparedoxin peroxidases. Recombinant TXNPxs reduce both H_2O_2 and peroxynitrite. Based on those observations, we have generated *L. infantum* parasites over-expressing the different TXNPx, two cytosolic and one mithocondrial, to test if they would also detoxify peroxynitrite in the context of the parasite. Here, we show that this is indeed the case, mainly in what refers the cells over-expressing the cytosolic enzymes. To see whether these TXNPxs would play a role in parasite resistance to macrophage produced ROS/RNS, experiments are underway comparing the capacity of the different parasite lines to infect macrophages.

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Up-regulation of the NADPH oxidase Nox4 by TGF-beta in hepatocytes: Correlation with its pro-apoptotic activity

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TGF-β belongs to a family of growth factors that play important roles in growth control, development and differentiation. In foetal rat hepatocytes, TGF-β inhibits growth and induces apoptosis by a mechanism dependent on oxidative stress, mitochondrial release of cytochrome c and caspase activation. Due to the implication of ROS in triggering apoptosis, we have analysed the expression of genes involved in this process, such as different members of the *nox* family that are expressed in hepatocytes. Among them, *nox4* expression and ROS generation are strongly correlated with the pro-apoptotic TGF-β activity. Analysis of regulation of *nox4* gene expression by TGF-β has shown to increase markedly, in a time- and dose-dependent manner, both by semi-quantitative and quantitative PCR. The *nox4* induction by TGF-β requires TGF-β receptor I activation, since incubation with the receptor inhibitor SB431542 completely blocks such induction. Cloning of 1,1 kb of *nox4* promoter and transfection studies with vectors containing this genomic region coupled to the luciferase reporter gene have shown a significant expression increase in luciferase activity when cells are incubated with TGF-β. Search of SMAD binding elements (SBE) in this region indicated the presence of several putative SBE, whose characterization is under study. Finally, experiments with *nox4* siRNAs have shown that already when a 30–50% of *nox4* is silenced, cells are protected to a similar extent from the apoptosis induced by TGF-β with concomitant decreases in NADPH oxidase activity and caspase-3 activation and protection of cell death.

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Evaluation of glutathione metabolism in cultured rat hepatocytes during acetaminophen injury

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Acetaminophen (AAP) is a safe analgesic and anti-pyretic drug when used at therapeutic doses. On the other hand, the AAP overdose is one of the most frequent causes of drug-induced acute liver failure. Albeit the mechanisms of AAP injury have been extensively studied recently, some of them remain to resolve. Glutathione is an essential compound acting in detoxification of AAP because it binds to the active metabolite, N-acetyl-p-benzoquinone imine (NAPQI). After depletion of GSH levels, NAPQI binds to a number of cellular proteins. The aim of our study was to evaluate the changes in glutathione metabolism, GSH and GSSG levels and glutathione reductase (GR) activity, during AAP injury. Especially, we focused on time course of changes with respect to increasing AAP doses. Hepatocytes were isolated from male Wistar rats by collagenase perfusion and cultivated on collagen-coated 24-well plates. Our results proved that the activity of GR is evidently decreased in relation to AAP concentration and time of incubation

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(after 24 h incubation with AAP 5 mM, 10 mM and 20 mM, respectively—activity of GR compared to controls: 60 ± 6%, 36 ± 5% and 21 ± 7%, respectively).

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Protein kinases activity in different fractions of gastric mucosa during the stress ulceration

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In spite of more than a century of exploration, reasons and mechanisms of this disease are still understood incompletely. The goal of our research was to explore the role of protein kinases system in plasma membrane and cytosol of gastric mucosal cells in molecular mechanisms of ulcer development. White Wistar rats of both sex 130–150 g weight were used during the experiment. Social stress (Groisman, Carevina) was used for ulcer development. Cyclonucleotide-, calcium- and phospholipid-dependent protein kinases activity was evaluated by insertion of $^{32}P_H$ in H2B histone. Tyrosine protein kinase activity was evaluated by insertion of $^{32}P_H$ in angiotensin ll. Radioactivity was evaluated in toluene scintillator on counter Delta-300 USA. Our exploration shows that serine-threonine systems of tyrosine phosphorylation have different sensitivity to membrane pathological state that is observed during the ulceration. Due to obtained results, cyclonucleotide-, calcium- and phospholipid-dependent protein kinases activity in cytosol of gastric mucosa changed insignificantly against control. Tyrosine protein kinase activity increased by 1.5-times. In plasma membrane fraction, increased activity of all explored protein kinases was observed. Increased activity of tyrosine protein kinase causes misbalance in normal cells growth system, which causes theirs transformation. So violations in interactions between EGF and its membrane receptors take place. EGF regulates gastric mucosal epithelial cells regeneration. Changes in its secretion lead to violations of HCl secretion. The obtained data show that protein kinases are sensitive chain in signal transduction cascades during the ulceration and they are involved in metabolic pathways which are used during the ulceration. Violations in these pathways may lead to development of this pathology.

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Plasma membrane-bound cytochrome b_5 reductase forms a large network of redox centres that co-localizes with cholera toxin B binding sites in cerebellar granule neurons in culture

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Redox centres play a major role in neuronal defence against oxidative stress and survival. In cerebellar granule neurons in culture (CGN) a large pool of the flavoproteins are associated with the plasma membrane [1]. During CGN maturation there is a nearly 2-fold increase of the intensity of green autofluorescence which displays a temporal evolution that correlates with the levels of expression of cytochrome b_5 reductase. Fluorescence resonance energy transfer (FRET) imaging from flavines fluorescence to plasma membrane-bound lipid acceptors revealed a clustered distribution of flavoproteins associated with the plasma membrane of CGN. Furthermore, we have found a large FRET efficiency between the 'lipid raft' marker cholera toxin B (labelled with Alexa-488) and anti-cytochrome b_5 reductase stained with a secondary Cy3 antibody in CGN fixed with parafolmaldehyde. Our results of FRET imaging shows that plasma membrane-bound cytochrome b_5 reductase is located at less than 15 nm from cholera toxin B binding sites. Moreover, CGN pre-treatment with anti-cytochrome b_5 reductase produced a decrease of cholera toxin B binding sites of 0.4 picomoles/600 000 cells or $\approx 4 \cdot 10^5$ binding sites per cell, and this is fully consistent with Cb_5R as a major flavoprotein component of the CGN plasma membrane. In summary, our study unravels that cytochrome b_5 reductase forms a major network of redox centres largely enriched in the CGN neuronal soma and associated with plasma membrane 'lipid rafts'.

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Reference

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Dietary thioproline improve mitochondrial and brain function and survival in mice

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Male mice on a diet supplemented with thioproline (*l*-thiazolidine-4-carboxylic acid), at 2.0 g/kg of food from 28 weeks of age and for mice entire life, showed a 29–23% increased median and maximal life span. These survival increases were associated with improved neurological functions. Compared to control mice, thioproline-supplemented mice had a 20% lower integral spontaneous food intake and 10% lower body weight at 100 weeks of age. Body weight showed a statistically significant inverse relationship with survival and neurological performances. Thioproline-supplemented mice exhibited a 58–70% decrease of the age-dependent oxidative damage in brain and liver mitochondria at 52 weeks (old mice) and 78 weeks (senescent mice) of age, respectively. The age-associated decrease of brain mitochondrial enzyme activities, NADH-dehydrogenase, cytochrome *c* oxidase and mitochondrial nitric oxide synthase (mtNOS), in old and senescent mice were markedly prevented (51–74%) by thioproline. Conditions that increase survival, as dietary thioproline, moderate physical exercise, vitamin E dietary supplementation and high spontaneous neurological activity, ameliorate mitochondrial dysfunction in aged brain and liver. The prevention of the mitochondrial dysfunction is likely to improve mitochondrial biogenesis and turnover mediated with the pleiotropic signalling of H_2O_2 and NO diffusion to the cytosol.

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Assessment of lipid peroxidation and antioxidant status in kidneys and liver of rats treated with sulphasalazine

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Sulphasalazine (SASP) is a drug used in the treatment of ulcerative colitis and in Crohn's disease. The aim of the study was to investigate the modifications in endogenous antioxidant capacity and oxidative damage in livers and kidneys of rats treated with SASP. Adult male rats were orally administered with 0 or 600 mg SASP/kg body weight in divided doses for 14 days followed by a 2-week period without treatment. Animals were killed on day 15 or 29 and kidneys and liver were removed and processed to examine the following stress markers: reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase (GR), glutathione peroxidase (GPx), glutathione transferase (GST), superoxide dismutase (SOD), catalase (CAT) and thiobarbituric acid reactive substances (TBARS). Histological examination of kidneys showed megamitochondrions in rats treated with SASP after a 2-week period without administration. Dropsical degeneration was also observed in this group. In kidney tissue, the TBARS levels decreased significantly as a result of SASP treatment. Following 2 weeks without administration, a tendency to restore these levels was noted. CAT activity was significantly restored after a period of 2 weeks without SASP. In liver, while SOD activity was significantly reduced after 2 weeks without treatment, TBARS levels were enhanced. SASP administration caused a significant decrease of CAT activity that was partially restored after a 2-week period. It is suggested that the effects of SASP in kidneys and liver, at least partially, is a consequence of oxidative damage.

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Inhibition of the MAPK/ERK pathway potentiates TGF-beta-induced apoptosis in Hepg2 cells: Role of oxidative stress

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Transforming growth factor-beta (TGF-β) induces apoptosis and cell cycle arrest in hepatocytes. However, many human hepatocellular carcinoma (HCC) cells do not respond to the tumour suppressor signalling of this cytokine. We tried to understand the molecular mechanisms that confer cell resistance to TGF-β induced apoptosis in HCC cells. We used the hepatoblastoma cell line HepG2 in this study. For apoptosis experiments, caspase-3 activity and DNA fragmentation were analysed. Oxidative stress was analysed fluorimetrically with specific probes for reactive oxygen species (ROS) and glutathione. Protein levels and/or activation were visualized by Western blot. mRNA levels were analysed by semi-quantitative-PCR and Multiplex-Ligation-dependent-Probe-Amplification. For specific gene knockdown, siRNA was used. The inhibition of the MAPK/ERK pathway (which is over-activated in these cells) by PD98059 enhances TGF-β-induced apoptosis, which correlates with increased ROS levels. Moreover, this co-treatment induces *bmf* expression and decreases *bcl-xL* and *mcl-1* levels, pro- and anti-apoptotic members of the *bcl-2* gene family. The use of antioxidants such as GEE (Glutathione-Ethyl-ester) and DPI (diphenyleneiodonium) blocked partially or completely, respectively, ROS production, caspase-3 activity, up-regulation of *bmf* and down-regulation of *bcl-xL*. Because DPI is a general flavoprotein inhibitor, we studied whether NADPH oxidase (Nox) family is involved in the TGF-β-induced apoptosis. We could only detect an induction of Nox4 when TGF-β and PD98059 treatment was used. Furthermore, Nox4 knockdown impaired ROS increase and caspase-3 activation. In conclusion, inhibition of the MAPK/ERK pathway in HepG2 sensitizes to TGF-β-induced apoptosis, which correlates with induction of *nox4* expression and oxidative stress-mediated regulation of *bmf*, *bcl-xL* and *mcl-1*.

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Studies on effects of seikosaponins from bupleurum fruticescens on human leukaemic cells

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The pharmacologic strategies for cancer treatment are designed for promoting cell death by apoptosis, in order to achieve tumour cell eradication and to avoid cell growth and expansion. Testing new anti-tumour agents, seikosaponins (triterpenic saponins), extracted from *Bupleurum* species show, among others, anti-proliferative activity and induction of cell differentiation, which implies cell cycle arrest and apoptosis. We are assaying different extracts from *Blupeurum fruticescens* looking for new agents with higher efficacy and selectivity towards tumour in comparison to normal cells. To understand the anti-tumour mechanism of the compounds present in these extracts, we are studying their effect on two human leukaemia cell lines: K562 (chronic myeloid leukaemia in blastic crisis), which is very resistant to apoptosis, and NB4 (acute promyelocytic leukaemia). Both cell lines showed a different behaviour. Buthanolic and dichloromethanolic extracts, used independently, affect cell viability of NB4 cells which are particularly sensible to the dichloromethanolic extract at a concentration of 20 µg/mL or higher, whereas K562 cells did not show sensibility to any of the investigated extracts. A possible relationship between the cytotoxic (anti-tumour) activity of these compounds and redox balance is also currently being investigated in our laboratory.

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Real time *in vivo* measurement of nitric oxide concentration dynamics in the rat hippocampus

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In the hippocampus, the production of $^{\bullet}$NO following stimulation of NMDA glutamate receptor has been implicated in synaptic plasticity, learning and memory as well as in neurodegeneration and disease. The measuring of $^{\bullet}$NO in real time has been a challenging task due to its physicochemical properties, reactivity and low concentration usually found in biological tissues. The use of electrochemical techniques with modified microelectrodes constitutes one of the most advantageous analytical approaches for monitoring the concentration dynamics of $^{\bullet}$NO *in vitro* and *in vivo* due to its high spatial and temporal resolution. We have used carbon fibre microelectrodes coated with Nafion and *o*-phenylenediamine (*o*-PD) for measuring $^{\bullet}$NO concentration dynamics in the hippocampus of living rat. The analytical performance of microelectrodes was assessed in terms of sensitivity (325 ± 34 pA/µM; $n = 14$), detection limit (20 ± 5 nM; $n = 14$) and selectivity ratios against major interferents, ascorbate ($> 10\,000$:1; $n = 14$), nitrite (5077 ± 1480:1; $n = 13$) and dopamine (134 ± 22:1; $n = 14$). The characterization of *in vivo* signals by electrochemical and pharmacological verification, permitted to ascribe the measured oxidation current to changes in $^{\bullet}$NO concentration in brain extracellular space. By inserting the microelectrode in CA1 and CA3 sub-regions of the hippocampus, a local stimulation with 20 mM glutamate (1–2 s, 10–15 psi) evoked transient $^{\bullet}$NO signals that correspond to an averaged $^{\bullet}$NO concentration increase of 615 ± 131 nM ($n = 15$) for a first stimulation followed by a desensitization-type phenomena, the later being dependent of individual peak duration (occurring for signals with half-width > 30 s). Moreover, the diffusion of $^{\bullet}$NO through the brain matrix was studied by local application of exogenous $^{\bullet}$NO to gain insights on the mechanisms of its removal/degradation.

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Testing the mitochondrial free radical theory of ageing by decreasing the mitochondrial ROS production

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Nowadays, the Mitochondrial Free Radical Theory of Ageing (MFRTA) is the most supported one to explain ageing. Unfortunately, most experimental data supporting such a theory are correlative; for example, long-lived animals produce fewer free radicals than short-lived ones. Thus, caloric restriction (CR) increases mean and maximum life span and decreases mitochondrial free radical production. However, CR also changes other parameters (e.g. insulin signalling) so it is not possible to attribute exclusively the increase in longevity to changes in free radical production. In fact the only definitive way to check MFRTA is to specifically decrease mitochondrial free radical production and, in parallel, study the effect of such a modification on longevity. If MFRTA is correct the reduction in mitochondrial free radical production must increase mean and maximum life span. As an example of such an approach we have created a Drosophila fly model that produces fewer mitocondrial reactive oxygen species (mtROS) without altering mitochondrial oxygen consumption. As opposed to MFRTA's predictions, flies producing fewer free radicals do not live longer.

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Evaluation of the relative antioxidant capacity of wine samples and pharmaceutical formulations by sequential injection analysis

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Nowadays it's well known that the ingestion of substances with antioxidant activity can be important in the prevention of oxidative stress and consequently in the prevention of some health disorders [1]. Many studies have been published proving the benefits of ingesting moderate amounts of red wine in the prevention of coronary diseases, so that in the last decade several works related with the evaluation of the antioxidant capacity of wine have been developed [2]. On the other hand, studies showed that the therapeutic effect of some anti-inflammatory drugs may also be due to its scavenging activity towards free radicals produced in inflammatory reactions, as a result of its antioxidant properties, and so to the ability of protecting the biological

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systems from the harmful effects of oxidative processes [3]. The ABTS assay is one of the most used methods for the evaluation of the antioxidant capacity. It is based on the reduction of the pre-formed ABTS monocation radical ($ABTS^{\cdot+}$), in the presence of hydrogen donator antioxidant substances, with a decrease in the initial absorbance of the solution at 734 nm [4]. The present work describes the automation of the ABTS assay in sequential injection analysis (SIA) systems, in order to evaluate the antioxidant capacity of wines and etodolac pharmaceutical formulations. Automation resorting to SIA systems presents several advantages like versatility, robustness, sample and reagents economy and reduced residues production [5]. It is a powerful and versatile instrumental sample handling approach that provides automation with relatively simple instrumentation.

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Acetaminophen inhibits prostanoid synthesis by scavenging the PGHS-activator peroxynitrite

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The primary pharmacological target of acetaminophen is prostaglandin endoperoxide H_2 synthase (PGHS). The enzyme is comprised of a radical-based catalytic mechanism, which is initiated and maintained by the persistent presence of peroxides, particularly peroxynitrite, and summarized by the term 'peroxide tone'. In addition to the prevalent concept, which assumes a direct reduction of the active, oxidized enzyme, we propose that acetaminophen is a potent scavenger of peroxynitrite. Nanomolar concentrations of peroxynitrite increased the activity of isolated PGHS and prostacyclin formation by aortic endothelial cells. This elevated activity was efficiently inhibited by pharmacologically relevant concentrations of acetaminophen and free radical scavengers. However, when other peroxides such as H_2O_2 or tert-butyl-OOH provided the peroxide tone, acetaminophen demonstrated only negligible inhibitory effects. Our concept could help to explain the efficacy of acetaminophen in cell types with moderate oxidant formation. Moreover, inflammatory sites are commonly associated with high levels of peroxynitrite. This might overwhelm acetaminophen's ability to effectively decrease peroxynitrite to an extent that significantly reduces PGHS activation. The concept presented herein provides a molecular basis to explain the excellent analgesic and anti-pyretic properties of acetaminophen aside from its marginal anti-inflammatory effects.

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Metformin reverts vascular dysfunction in hyperlipidemic Goto–Kakizaki rats

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Metformin hydrochloride, a biguanide, improves the insulin-resistant state by unknown mechanisms, reducing gluconeogenesis and enhancing peripheral glucose uptake. As a result, metformin hydrochloride promotes reduction of the plasma glucose levels. Despite the action of metformin the precise mechanisms by which this drug prevents the development of atherosclerotic disorders have not yet been fully elucidated. This study was undertaken to test the hypothesis that hyperglycaemia induces the generation of reactive oxygen species and that the oxidative stress thereby exerted is diminished by treatment with metformin. As a model of type 2 diabetes we used Goto–Kakizaki (GK) rats fed with an atherogenic type of diet (GKAD). In parallel with the development of diabetes (glycaemia and insulin resistance indexes), the generation of oxidative stress was determined by measurement of lipid peroxides (8-isoprostanes), oxidized proteins (carbonyl activity) and 8-hydroxy-2′-deoxyguanosine (8-OHdG). Vascular activity was determined in aorta by measuring the endothelium-dependent vasodilation in response to acetylcholine and vasoconstriction in response to phenylephrine. GK rats with hyperlipidaemia showed increased oxidative stress and impaired endothelial dependent vasodilation. Metformin did not improve the oxidative stress parameters evaluated but completely reverted endothelial function in the GKAD rats. These observations provide *in vivo* evidence that systemic oxidative stress does not seem to be involved in the beneficial effects observed with metformin that completely prevent vascular dysfunction in GK rats with hyperlipidaemia.

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Glyclazide improves anti-oxidant status and nitric oxide-mediated vasodilation in type 2 diabetes

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Diabetes mellitus is characterized by oxidative stress, which in turn determines endothelial dysfunction. Gliclazide is a sulphonylurea antidiabetic drug with antioxidant effect due to its azabicyclo-octyl ring. In streptozotocin-induced diabetic rats, gliclazide prevented endothelial dysfunction when given orally and improved the impaired relaxations to exogenous nitric oxide (NO) when applied on aortic segments. We hypothesized that gliclazide may have a beneficial effects on endothelial function in Goto-kakizaki rats (GK) and animal model of type 2 diabetes with induced dyslipidemia. GK diabetic rats fed with an atherogenic type diet (GKAD) were treated with gliclazide (GK+ gliclazide) after 3 months of the dyslipidemia induction. We therefore evaluated the influence of gliclazide on both metabolic and oxidative status and NO-mediated vasodilation. GKAD rats showed increased oxidative stress and impaired endothelial dependent vasodilation. GK+ gliclazide showed increase sensitivity to NO-mediated vasodilation, a significant decrease in fasting glycemia and also a significant decrease in urinary 8-hydroxy-2′-deoxyguanosine levels. In conclusion, our results demonstrate that glicazide treatment improves both antioxidant status and NO-mediated vasodilation in diabetic GK rats with dyslipidemia. The availability of a compound that simultaneously decreases hyperglycemia, restores insulin secretion and inhibits oxidative stress produced by high glucose seems to be an interesting therapeutic prospect for the prevention of vascular complications of diabetes.

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Atorvastatin and insulin in vascular dysfunction in Goto–Kakizaki rats

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It has been repeatedly proven that statins improve endothelial function in diabetes. Insulin has also important vascular actions. It stimulates production of nitric oxide from endothelium, which leads to capillary recruitment, vasodilation, increased blood flow and subsequent augmentation of glucose disposal in classical insulin target tissues. The mechanisms of action for statins and insulin are distinct. Therefore, we conducted a study in which the effects of atorvastatin and insulin therapies alone or in combination on both endothelium-dependent vascular reactivity and biochemical parameters were compared in Goto–Kakizaki (GK) rats, a model of type 2 diabetes, with hyperlipidaemia. In parallel with the development of diabetes (glycaemia and insulin resistance indexes), the generation of oxidative stress was determined by measurement of lipid peroxides (8-isoprostanes), oxidized proteins (carbonyl activity) and 8-hydroxy-2′-deoxyguanosine

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(8-OHdG). Vascular activity was determined in aorta by measuring the endothelium-dependent vasodilation in response to acetylcholine and vasoconstriction in response to phenylephrine. GK rats with hyperlipidaemia showed increased oxidative stress and impaired endothelial dependent vasodilation. Atorvastatin and insulin therapies in combination decreased generation of reactive oxygen species and improved vascular dysfunction in GK rats with hyperlipidaemia. Thus, combination therapy has beneficial effects on endothelial function in GK rats with hyperlipidaemia.

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HNE induces UCP2 expression and proton leak to limit oxidative stress but reduces ATP synthesis in non-alcoholic steatohepatitis

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Uncoupling Protein-2 (UCP2), mediating proton leak is able to limit ROS production. It has been reported *in vitro* that HNE activates UCP2. We studied the role of mitochondrial oxidative stress on the expression of UCP2, proton leak and H_2O_2 synthesis in a rat model of NASH and its possible effect on ATP homeostasis. NASH was induced in Wistar rats by High Fat/Methionine-Choline Deficient diet. Animals were sacrificed after 3, 7 and 11 weeks of diet. Mitochondrial function (RCR, membrane potential and proton leak), UCP2 mRNA expression, H_2O_2 synthesis, HNE-protein adducts, ATP content and ATPase activity were measured in isolated mitochondria from NASH and SHAM rats. UCP2-dependent proton leak increased at 7 and 11 weeks of NASH development, together with HNE-protein adducts and UCP2 expression. H_2O_2 production significantly increased at 7 weeks and did not change at 11 weeks. ATP content decreased with time, but ATPase activity was unaffected by NASH. Our data show, *in vivo*, that NASH induces mitochondrial over-production of ROS and oxidative stress. HNE induces activation of UCP2 and over-expression of the UCP2 mRNA to increase proton leak, allowing mitochondria to set up the substrate oxidation and lower redox pressure during fat accumulation, limiting oxidative stress. Despite these favourable effects, UCP2 reduces ATP synthesis, by uncoupling the respiratory chain from complex V activity, and exposes the hepatocytes to chronic ATP depletion. This may increase the susceptibility of the liver to noxious stimuli that requires energy supply, i.e. ischemia/reperfusion injury or liver regeneration.

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Protective role of antioxidants and the Gardos channel activation against human erythrocyte oxidative haemolysis

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Circulating erythrocytes (Es) are always subject to oxidative attacks. tert-Butyl hydroperoxide (t-BHP) is often used as a model substance to study mechanisms of oxidative damage in Es. The short-term (less than 1 h) incubation of Es with t-BHP decreases the concentration of reduced glutathione, induces haemoglobin oxidation and alters ion permeability of cell membranes. As shown in our earlier study, incubation with t-BHP for 20 min at 37°C results in E swelling (cell density and osmotic resistance significantly decrease). This effect diminished considerably in the Ca^{2+}-containing medium suggesting the involvement of Gardos channel activation in the oxidant-induced changes of the volume-related characteristics of Es [1]. More prolonged incubation with different concentrations of t-BHP may result in lysis of Es [2]. In this study, it was shown that E incubation with t-BHP (1–3 mM) for more than 2 h at 37°C causes concentration- and time-dependent haemolysis. Antioxidants quercetin and taxifolin (10–50 μM) are protected Es from haemolysis by t-BHP. When the antioxidants tested were present in the medium at the time of the addition of the t-BHP the protection was much greater than when they were added 15–30 min later. The haemolysis was independent on calcium presence in medium but increased significantly in high-potassium medium and in the presence of Gardos channel inhibitor clotrimazole. These results demonstrate the protective action of some flavonoide antioxidants and Gardos channel activation against E lysis under conditions of t-BHP-induced oxidative stress.

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Chemiluminescent monitoring of hydroxyl radical scavenging by mannitol in a SIFA system

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Myoglobin, a small and stable metalloprotein, has the ability to, when in the ferric state (Fe^{III}) and in alkaline medium, oxidize luminol. As a consequence, myoglobin is reduced to its ferrous state (Fe^{II}) while luminol yield an excited singlet state of the aminophthalate ion that is responsible for the emission of light. Luminol oxidation by myoglobin can be explained by radical production since luminol is known to generate light in the presence of free radicals [1]. These radicals could be produced in a redox cycle of the metalloprophyrin in which reduction of Fe^{III} to Fe^{II} results in hydroxyl radical ($OH^{\bullet}$) formation from OH^-. Mannitol is a polyol (sugar alcohol) with a strong hydroxyl radical scavenger activity. Therefore, it has the ability to inhibit the luminescence response generated upon reaction of myoglobin and luminol, a property that could be used for its determination in samples with distinct origins. In this work a single interface flow analysis (SIFA) system [2] was implemented for the chemiluminescent monitoring of mannitol levels in food, pharmaceutical and biological samples. SIFA systems area a recent concept in flow analysis and are based on the establishment of a single reaction interface that puts in contact the sample and reagent solutions. One of the main advantages of SIFA systems is that they no longer rely on the utilization of well-defined and compelling sample and reagent volumes, which facilitate system configuration and resulted in enhanced simplicity and operational versatility.

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Plasma nitrite concentration in obese and in normal weight subjects

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Obesity has been implicated as a major cause of cardiovascular diseases and is considered a low grade systemic inflammatory state, as demonstrated by increased levels of high sensitivity-C-reactive protein (hs-CRP) found in the obese population. Nitric oxide (NO) plays a central role in maintaining vascular homeostasis and changes in plasma nitrite (NO_2^-) have been used to evaluate vascular NO production. Reduction in the bioavailability of vascular NO is believed to contribute to endothelial cell dysfunction associated with obesity. Common ELISA methods for plasma NO bioavailability can not distinguish between nitrite (form of plasma NO storage) and nitrate (also derived from common foods). Therefore, NO bioavailability was investigated in 50 obese (BMI 34.05 ± 0.64 kg/m^2) and 50 normal weight (BMI 24.03 ± 0.38 kg/m^2) subjects by determining plasma nitrite by a highly sensitive HPLC method. In the same subjects hs-CRP was measured as inflammatory marker. Obese show significantly lower plasma nitrite than normal weight subjects (273 ± 20 nM vs 357 ± 29 nM, $p < 0.01$)

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and higher hs-CRP levels (respectively, 3.47 ± 0.37 mg/dL vs 1.44 ± 0.23 mg/dL, $p < 0.01$). The finding that obesity is associated with decreased plasma nitrite is consistent with the hypothesis that NO storage (i.e. nitrite) can diminish in inflammatory states; besides, plasma nitrite could be a useful marker to monitor the degree of endothelial cell dysfunction associated with obesity.

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P-170

Structural change of malvidin 3-glucoside during wine ageing produces a new portisin with a remarkable antioxidant potency

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Anthocyanins are a sub-class of polyphenols responsible for the colour of red wines, which is a major attribute of wine quality. Also, they are thought to greatly contribute to the protective effects of regular and moderate wine consumption to cardiovascular diseases (CV), partially by preventing LDL from oxidative damage. During ageing, the wine colour alters, mainly due to the structural changes of anthocyanins. Recently, a new class of blue pigments (portisins) resulting from the reaction between the pyruvic derivative of malvidin 3-glucoside and catechin, mediated by acetaldehyde, was reported in Port wine and structurally characterized. Thus, the aim of this study was to evaluate the antioxidant activity of the portisin derived from Mv3glc, in comparison with Mv3glc and its aglycon (Mv), by using *in vitro* models, namely LDL oxidation promoted by AAPH-generated peroxyl radicals and peroxynitrite-mediated dihydrorhodamine (DHR) oxidation. These compounds, in very low concentrations, inhibit either LDL oxidative damage, in terms of conjugated dienes formation or peroxynitrite-mediated DHR. In both assays, portisin shows a remarkable strong potency while Mv exhibits the lowest activity, pointing to the relevance of glycosylation and to the flavanol moiety in the molecule. These data indicate that portisin, putatively formed by structural changes of Mv3glc during wine storage, may contribute to its beneficial properties in the context of CV prevention. Ongoing studies aim to evaluate other potential benefits of this compound.

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Transforming growth factor-β1 modulates expression of macrophage migration inhibitory factor and heme oxygenase-1 expression in aortic smooth muscle cells

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Anti-inflammatory properties of transforming growth factor-β1 (TGF-β1) may account for its protective role against atherosclerotic plaque rupture. The transcription factor Nrf2 mediates induction of cytoprotective genes such as heme oxygenase-1 (HO-1) which catabolizes the pro-oxidant heme to generate the vasodilator carbon monoxide and antioxidants biliverdin and bilirubin. Macrophage migration inhibitory factor (MIF) exhibits catalytic thiol-protein oxidoreductase activity and plays a role in redox homeostasis and inflammatory responses. The present study investigates whether TGF-β1 induces HO-1 and MIF expression in cultured mouse and human aortic smooth muscle cells (SMC). Western blot analyses revealed that HO-1 and MIF expression were significantly enhanced by TGF-β1 (0–10 ng/ml, 0–12 h, $p < 0.01$, $n = 5$) in cells cultured from wild-type but not Nrf2 deficient mouse aortas. Induction of HO-1 in human SMC by TGF-β1 (5 ng/ml, 12 h) was significantly ($p < 0.05$, $n = 3$) attenuated by superoxide dismutase (200 U/ml) or following inhibition of NADPH oxidase activity by diphenylionium (2 μM). TGF-β1 (0–10 ng/ml, 2 h) elicited significant nuclear translocation of Nrf2 and attenuated accumulation of the pro-apoptotic mediator p53 in response to hydrogen peroxide generated by glucose oxidase (10 mU/ml, 2 h) in human SMC. Our findings suggest that TGF-β1 may afford protection in aortic SMC by enhancing Nrf2 mediated induction of HO-1 and MIF during oxidative stress in atherogenesis.

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Kinetics of hypobromous acid-mediated oxidation of lipid components and antioxidants

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Hypohalous acids are generated from the oxidation of halide ions by myeloperoxidase and eosinophil peroxidase in the presence of H_2O_2. These oxidants are potent antibacterial agents, but excessive production can result in host tissue damage, with this implicated in a number of human pathologies. Rate constants for HOCl with lipid components and antioxidants have been established. Here the corresponding reactions of HOBr have been examined to determine whether this species shows similar reactivity. The second-order rate constants for the reactions of HOBr with 3-pentenoic acid and sorbate, models of lipid double bonds, are 1.1×10^4 and 1.3×10^3 M^{-1} s^{-1}, respectively, while those for reaction of HOBr with phosphoryl-serine and phosphoryl-ethanolamine are $\sim 10^6$ M^{-1} s^{-1}. Second-order rate constants (M^{-1} s^{-1}) for reactions of HOBr with Trolox (6.4×10^4), hydroquinone (2.4×10^5) and ubiquinol-0 (2.6×10^6) were determined, as models of the lipid-soluble antioxidants, α-tocopherol and ubiquinol-10; these rate constants are ~ 50–2000-fold greater than for HOCl. In contrast, the second-order rate constants for reaction of HOBr with the water-soluble antioxidants, ascorbate and urate, are $\sim 10^6$ M^{-1} s^{-1} and similar in magnitude to those for HOCl. Kinetic models have been developed to predict the sites of HOBr attack on low-density lipoproteins. The data obtained indicate that HOBr reacts to a much greater extent with fatty acid side chains and lipid-soluble antioxidants than HOCl; this has important implications for HOBr-mediated damage to cells and lipoproteins.

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Study of antioxidative properties of HSA on LDL oxidation accompanied by MALDI lipid profiling

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LDL oxidation plays a significant role in the pathogenesis of atherosclerosis, therefore the inhibition of this process is of great importance. Human serum albumin (HSA), which is postulated to have antioxidant potential, was investigated concerning its effects on copper-mediated LDL oxidation *in vitro*. HSA purified from plasma without any additives and containing stabilizers (0.48 mg/ml caprylate and 0.82 mg/ml N-acetyltryptophan) (Octapharma Pharmazeutika) was used. IgG isolated from plasma was used as a reference protein. The oxidation process was monitored by the thiobarbituric acid reactive substances assay (TBARS) and lipids were analysed in parallel by MALDI mass spectrometry (MALDI lipid profiling). Based on TBARS assay, LDL oxidation between 2–24 h was inhibited by 100 vs 95% in the presence of HSA, while inhibition by IgG was clearly less effective (34 vs 0%), respectively. In the presence of HSA containing stabilizers, LDL was protected from oxidation by $\sim$ 100% over the whole incubation time. MALDI analysis revealed that degradation of phosphatidylcholine (PC) containing linoleic (18:2) or arachidonic (20:4) acid was inhibited by 64–96% in the presence of HSA but only 22–51% by IgG. Lyso-PC formation was reduced by 41–52% in the presence of HSA but no protective effects were observed using IgG. Our investigation clearly outlines the antioxidative properties of HSA on LDL oxidation and similar effects might be anticipated *in vivo* too. Stabilizers even slightly enhanced protection of LDL from oxidation. MALDI lipid profiling allowed monitoring of lipid degradation during the LDL oxidation process directly at the molecular level.

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Post-prandial erythrocites antioxidant status. Influence of a lipid rich meal

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Increasing evidence suggests that post-prandial state is a contributing factor to the development of atherosclerosis. Human individuals by eating regular meals are predominantly in a post-prandial state throughout the day. so we have considered it of interest to study biochemical parameter modifications in post-prandial state after a fat rich meal in order to better understand the mechanisms linking these modifications to atherosclerosis and cardiovascular pathology. Oxidative stress is a hallmark of atherosclerosis so we have also looked at variations of oxidative stress parameters We have chosen a standard fat load (70 g fat and 30 g bread), taking into consideration that typical Western diets which are also becomming typical for the Romanian population are rich in fats, causing hyperlipemia several hours a day. Thirty healthy human volunteers have been selected in the study. Venous blood has been collected before and from hour to hour during 6 h after administration of the meal. Plasma lipid (triglycerides, total cholesterol, LDL-cholesterol, HDL-cholesterol, total lipids) and glycemic profiles have been evaluated using standard biochemical methods. Erythrocites antioxidant status has been investigated measuring superoxide dismutase, catalase and glutathione peroxidase activity, thiols level and total antioxidant activity. The influence of the plasma lipid status on antioxidant parameters in erythrocites is discussed.

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The influence of Artemisia Absintium L. extracts on stability of human erythrocytes

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Free radicals are constantly formed in the body during normal metabolic processes and biological systems have evolved to live with them, control them and even utilize them. However, when their formation is greatly increased or protective mechanisms compromised a state of oxidative stress will result. Erythrocytes are a target of such damage, including itself and membrane lipids. The aim of this presented work is investigation of influence drug plant as anti-inflammatory natural antioxidant on level of lipoid peroxidation and stability of erythrocytes. As biological targets we used both erythrocyte and lipid suspension obtained from human donor blood. As drug plants we investigated ethanol extracts with different percentages of Artemisia Absintium L. Our obtained results show that 96% extracts of plant very quickly breaking off erythrocyte membranes and had lower level antioxidant activity than 30% ethanol extracts. A mathematical simulation of results allows us to solve the complete equations describing the stability erythrocyte membranes. The creation of such a model will be an important addition to the field of membrane lipid oxidation kinetics.

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Activation of the JNK pathway in the hippocampus during bacterial meningitis: Association with neuronal apoptosis?

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Brain damage in bacterial meningitis is characterized by apoptosis in the dentate gyrus of the hippocampus. The death of these cells is thought to be responsible for the learning and memory deficits often presented by patients surviving the disease. Apoptosis in bacterial meningitis has been proposed among other factors to be due to excessive release of glutamate and formation of reactive oxygen species. Studies in other models of acute brain injury such as cerebral ischemia have shown that activation of the redox-sensitive c-Jun NH_2-terminal kinase (JNK) pathway is responsible for hippocampal neuronal apoptosis, in particular the one induced by the neuron-specific JNK3 isoform. Whether JNKs are involved in hippocampal neuronal apoptosis due to bacterial meningitis has not been investigated so far. Here we show in a well-established infant rat model of pneumococcal meningitis that JNK3 and not JNK1/2 is activated in the hippocampus before the onset of apoptosis. Furthermore, JNK activation is accompanied by increased phosphorylation of its major down-stream target c-Jun. Immunohistochemically, increased c-Jun phosphorylation localizes to neurons in the inner rim of the hippocampal dentate gyrus, the primary site of apoptosis during bacterial meningitis. Using highly specific JNK inhibitors (e.g. D-JNKI-1), we are now investigating whether activation of the JNK pathway is actually responsible for hippocampal neuronal apoptosis in pneumococcal meningitis.

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Thiyl and sulphonyl radicals as catalytic agents for the cis-trans isomerization of unsaturated fatty acid residues in liposomes

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Sulphur-centred radicals (RS·) within the cells are engaged in a variety of redox, addition and abstraction reactions. There is increasing evidence to suggest that they are capable of initiating the chain of lipid peroxidation. Thiyl radicals undergo conjugation with molecular oxygen to generate the thiyl peroxyl (RSOO·) radical which can rearrange to thiyl-sulphonyl (RSO_2·) radical, another potent initiator of lipid peroxidation. These species are able to migrate from the aqueous to the membrane compartment and cause damage to lipids, converting cis-unsaturated fatty acid residues to corresponding trans isomers. The present study is focused on methanesulphonyl radicals (H_3CSO_2·), generated by one-electron reduction of methanesulphonyl chloride (H_3CSO_2Cl) in Ar-saturated aqueous solution. $H_3CSO_2Cl + e^-_{aq} \rightarrow H_3CSO_2\cdot + Cl^-$. In the presence of liposomes, H_3CSO_2· present in the water phase are able to diffuse through the membrane to isomerize the cis-oleoyl chain of the POPC (1-palmitoyl-2-oleoyl phosphatidycholine) into its trans isomer. The yield of cis/trans isomerization was investigated upon irradiation with gamma-rays in function of the lipophylicity of the sulphonyl radical. In N_2O-saturated solutions and in the presence of alcohols, methanesulphonyl radicals are still formed through the reaction with α-hydroxylalkyl-radicals. $ROH + HO^\bullet \rightarrow R^\bullet + H_2O$ (1); $H_3CSO_2Cl + R^\bullet \rightarrow H_3CSO_2\cdot + RCl$ (2). The reactivity of methanesulphonyl chloride with different alcohol-derivatives will be presented and discussed.

P-178

Vanadate and bone metabolism: Effect on proliferation and mineralization of fish bone-derived cells

Daniel M. Tiago[1], Vincent Laizé[2], M. Leonor Cancela[1], & Manuel Aureliano[3]

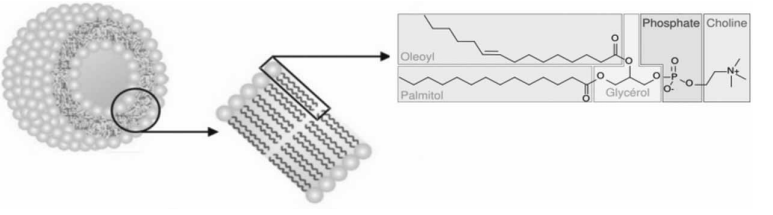

Figure 3.

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Vanadate is associated to several biological effects due to its capacity to regulate enzyme activities (e.g. phosphatases, ATPases, etc.). Insulin-mimicking, one of its key features, is mediated through specific inhibition of protein tyrosine phosphatases, which activates receptors tyrosine kinase and regulates signalling pathways: PI-3K (metabolic effects) and MAPK (growth). Bone formation, shown to be severely affected by vanadium deficiency or excess, was mainly investigated using mouse MC3T3-E1 and UMR106 cell lines and related to its insulin-mimetic properties. These studies, however, were rarely extended to other vertebrate systems and there is a lack of information concerning other models. Fish, however, are recognized as suitable models in vertebrate calcification investigation and vanadium higher abundance in seawater further confirms its suitability. Our goals were to investigate vanadate effects and mechanisms on growth and extracellular matrix mineralization of VSa13, a newly developed fish bone-derived cell line. We show that 5–7.5 μM metavanadate significantly stimulates proliferation, while strongly inhibits ECM mineralization (70–80%). Furthermore, insulin does not affect growth, but mildly inhibits ECM mineralization (25%). Insulin signalling involvement in these effects was studied using pathways inhibitors wortmannin and PD98059. Results indicate MAPK exclusive involvement in growth effects and putative PI-3K\ras\ERK pathway mediation of ECM mineralization effects. Moreover, partial reversion of metavanadate effects (65–85% mineralization recovery) and alkaline phosphatase lower measured activities in these conditions (30% decrease), but not with insulin, suggesting this enzyme involvement as an alternative target. VSa13 suitability in these studies was here demonstrated, but not always were previous observed effects in mammals confirmed.

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Actomyosin modulation by peroxynitrite

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Some pathological conditions in skeletal and cardiac muscle are associated with an oxidative stress-induced muscle injury. Additionally, previous reports have pointed out that several oxidants can directly alter contractile function by oxidative modification of myofibril proteins. In the present work we address the oxidative modifications accounting for the structural and functional impairment of the actin-filaments and the actomyosin complex under the oxidative stress mediated by peroxynitrite ($ONOO^-$), one of the most aggressive biological oxidant agents that has been implicated in a number of pathologies, including cardiac diseases and neurodegenerative processes. Experiments on purified protein have shown that $ONOO^-$ affects actin dynamics by inhibiting actin monomers (G-actin) polymerization and by producing an extensive depolymerization of the actin polymers (F-actin) leading to a complete block of the myosin ATPase activity stimulation. Furthermore, exposure of purified myosin to sub-micromolar $ONOO^-$ concentrations produced strong inhibition of the F-actin-stimulated myosin ATPase activity. The peroxynitrite-induced actomyosin impairment correlated with structural modifications that enhance the thermal instability of both actin and myosin, leading to partially unfolded states. The relevance of cysteines and methionines oxidation, 3-nitrotyrosine and carbonyl formation to actomyosin structural alterations induced by *in vitro* exposure to $ONOO^-$ has also been studied and the results suggest a major role for the highly reactive cysteines on actin and on myosin and also for some critical methionines on G-actin. Carbonyl and 3-nitrotyrosine formation do not seem to significantly contribute to the observed impairment. Finally, alterations of the actin cytoskeleton and the actomyosin system have been monitored in peroxynitrite-stressed cerebellum granule neurons and its potential cellular relevance evaluated.

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Pycnogenol®, French maritime bark extract, reduced symptoms of ADHD and modified level trasition metals

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Attention Deficit Hyperactivity Disorder (ADHD) is a neurodevelopment disorder of children. Pycnogenol®, consisting of phenolic acids, catechin, taxifolin and procyanidins, exhibits strong antioxidant properties through modulating of SOD, GPx and NOS activities *in vivo* and also shows chelating ability. The aim of the present study was to examine the effect of Pycnogenol® on ADHD symptoms and on the level of transition metals in the placebo-controlled, randomized, double-blind study. Sixty-one children were supplemented with 1 mg/kg/day Pycnogenol® or placebo over a period of 4 weeks. Symptoms of ADHD patients were examined by standard questionnaires evaluated by teacher (CAP, Child Attention Problems) and parents (CPRS, Conners Parent Rating Scale). Transition metals were determined by AAS. One-month Pycnogenol® supplementation causes a significant reduction of hyperactivity and improvement of attention of ADHD children evaluated by teachers and parents. In the placebo group no positive effects were found. One month after termination a relapse of symptoms was noted. We have found a decreased level of Zn in comparison to controls before the treatment. The concentration of Cu, Se and Fe was not different compared to the controls. The level of Cu was significantly reduced after 1 month of Pycnogenol administration. The other metals as well as ferritine and transferine activity were not influent by Pycnogenol. In summary,II II Pycnogenol® administration improves ADHD symptoms and modulated some transition metal levels.

P-181

Effect of nicotine during olfactory bulbectomy-induced depression and free radical production in brain

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This study evaluates the effects of nicotine (NIC) on bilaterally olfactory bulbectomy (OBX)-induced behaviour changes and oxidative stress. Male Wistar rats were divided into five groups as follows: (i) Control, (ii) Sham operated, (iii) OBX, (iv) NIC and (v) OBX + NIC. OBX was performed by a cranial window, the posterior edge of which was placed 5 mm anterior from the Bregma, and was created in the frontal bone. The olfactory bulbs were cut and aspirated, taking care not to cause damage to the frontal cortex. A period of 2 weeks allowed for the recovery from surgical procedure and the development of the OBX syndrome. NIC (1.5 mg/kg body weight) was administered intraperitoneally daily for 15 days, beginning after 2 weeks of the recovery. The oxidative stress by OBX was confirmed by a high level of lipid peroxidation products ($p < 0.001$) in brain, as well as by a decrease in GSH and antioxidative enzymes activities. Morevoer, OBX-induced hyperactivity in the open field following 15 days. These changes were reverted by NIC. In summary, these data indicate the beneficial effect of NIC characterized by a decrease of oxidative damage and a reduction of hyperactivity induced by OBX.

P-182

Modulation of c-fos gene expressions and glutathione levels by γ-glutamylcysteine ethyl ester in kainic acid model of neurodegeneration

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Many neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, stroke and epilepsy are linked to oxidative stress conditions. The understanding of the molecular pathophysiology of these brain dysfunctions will provide new clues that may lead to effective therapeutic approaches that will limit damage and slow cells. Recent studies showed that γ-glutamylcysteine ethyl ester (GCEE) has the ability to increase glutathione (GSH) levels in rat brain and displays antioxidant activity similar to GSH as asssessed by various *in vitro* indices such as hydroxyl radical scavenging and dichlorofluroescein fluorescence. Systemic administration of the glutamate analogue, kainic acid (KA), is a well-known model of excitotoxic neuronal cell death caused by prolonged seizures in adult rats. There is evidence that generation of reactive oxygen species and induction of an apoptotic pathway are involved in the mechanism of KA-induced neuronal damage. Although antioxidant properties of GCEE have been demonstrated, relatively little is known about their regulator effect on gene expression. According to our results, GCEE modulates the expression levels of c-fos in rat brain following KA-induced seizures. In addition, GCEE significantly increases GSH levels in brain against KA treatment. Therefore, we conclude that the regulation of immediate early gene response and glutathione status by GCEE may indicate its potential neuroprotective effect in KA-mediated neurodegeneration.

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FoxO3a mediates the PGC-1α transcriptional regulation of the mitochondrial antioxidant defense system

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Mitochondrial oxidative stress plays a mayor role in several common human pathologies, such as atherosclerosis, ischemia-reperfusion injury, cancer and neurodegenerative diseases. Recent studies have shown that transcriptional co-activator PGC-1α is directly involved in the transcriptional regulation of the mitochondrial detoxification system that protects cells against oxidative stress. PGC-1α interacts with DNA binding transcription factors (TF) that mediate its recruitment to the promoter regions of its target genes. We have sought to identify the TF that mediates the PGC-1α regulation of the mitochondrial ROS protection system. Our results suggest that this factor is FoxO3a, a positive regulator of stress resistance, cell cycle arrest and apoptosis genes. Experiments in endothelial cells and MEFs have shown that FoxO3a directly induces the complete mitochondrial detoxification system. In addition, we have demonstrated that FoxO3a and PGC-1α directly interact in endothelial cells and can be co-immunoprecipitated as a molecular complex. Importantly, we observed that PGC-1α up-regulation of mitochondrial detoxification genes is reduced in FoxO3a$^{-/-}$ MEFs. This co-regulation is likely to be dependent on the formation of a FoxO3a/ PGC-1α complex, since the mutation of the functional FOXO site in the MnSOD promoter abrogates the transactivation activity of PGC-1α on this gene. These results suggest that PGC-1α transcriptional regulation of stress genes is mediated by FoxO3a. We propose that PGC-1α and FoxO3a are key members of the complex that coordinately regulates this detoxification system. Further investigation of the functional interaction between FoxO3a and PGC-1α may provide significant insight into the mechanisms of cell response to oxidative stress and the life/death decision under stress conditions.

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Estradiol or genistein inhibits beta amyloid-induced inflammatory mediators in cultured astrocytes

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The neuropathological hallmarks of Alzheimer's disease (AD) are amyloid plaques, intra-neuronal tangles and the activation of glial cells. Glial swelling and astrogliosis are a characteristic response of astrocytes to inflammation and trauma, leading to the secretion of several potentially toxic products, including inflammatory mediators. Beta amyloid (Aβ) deposition can result in brain damage and neurodegeneration, but whether astrocyte activation participates in the Aβ-induced brain damage and its prevention by oestrogen or genistein are poorly known. Our study evaluates if Aβ is able to activate astrocytes to induce pro-inflammatory proteins such as cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) expression. Also we assess the prevention caused by oestradiol or genistein as well as the mechanism of action of these proteins. We used cultured astrocytes pre-treated with 17-β oestradiol or with genistein and 48 h later treated with Aβ. Our results show that Aβ (1–42) up-regulated iNOS and COX-2 expression and caused a significant increase in H_2O_2 levels in astrocytes in primary culture. However, in cells pre-treated with oestradiol or genistein, Aβ did not cause an increase in the H_2O_2 levels nor an up-regulation of the expression of iNOS and COX-2. In conclusion, our findings suggest that Aβ is able to induce inflammatory mediators in astrocytes and these effects are prevented by oestradiol or genistein.

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The protecting role of methionine-centred redox cycle proteins in nasal polyposis

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Nasal polyposis (NP) is an inflammatory condition, more frequently encountered among asthma and allergy patients. Its pathogenesis is multi-factorial. NP typically affects the middle and superior nasal meati along with the paranasal sinuses. Nasal polyps almost never involve the inferior nasal turbinate, the inferior nasal meatus or the nasal septum. The aim of the study was to investigate the levels and activities of methionine redox cycle proteins in nasal polyps, in order to evaluate the involvement of reactive oxygen-derived species (ROS) in NP. Thioredoxin, thioredoxin reductase and both methionine sulphoxide reductase isoforms were studied. Assays included Western blotting, HPLC and spectrophotometric analyses. Soft tissue homogenates from inferior turbinates of both NP patients and patients undergoing turbinectomy due to other reasons (non-NP patients) were used as controls. A noteworthy increase in proteins carbonyl content (PCC) was detected in nasal polyps, indicating an inflammatory process in the tissue. On the other hand, although the level of the proteins in nasal polyps remained unchanged compared to non-NP turbinates, a considerable rise in all analysed parameters was observed in NP turbinates. These results suggest that nasal polyps may secrete specific factors, which activate protective enzymes in adjacent tissues, in order to prevent spreading of the disease.

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Influence of different polyphenolic phytochemicals on prostanoid formation in HaCaT cells *in vitro* and *in vivo* using microdialysis

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UVB irradiation induces oxidative stress and inflammation in human skin. The role of different polyphenolic phytochemicals was tested for potential anti-inflammatory and antioxidative effects in skin and HaCaT keratinocytes. HaCaTs were irradiated with 100 mJ/cm^2 of UVB and incubated with increasing doses of oligomeric procyanidins, resveratrol, indole-3-carbinol, extracts from blueberry, pomegranate and ginkgo biloba (0.1, 1, 10, 100 and 1000 μM) for 22.5 h, 1.5 h after irradiation. For cutaneous microdialysis, areas of 3 cm^2 on the volar forearm of a young female volunteer were exposed to UVB irradiation (2-fold MED, 550 mJ/cm^2) and treated with amphiphilic cream containing 5% urea and 10% blueberry extract or 5% resveratrol or 5% oligomeric procyanidins all 2 h over 8 h under occlusion. Microdialysate samples were collected 22.5 h after irradiation at 30-min intervals up to 8 h. F_2-isoprostanes and prostaglandins were measured in cells, supernatants and microdialysate samples by sensitive gas chromatography-mass spectrometry. Addition of all phytochemicals

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caused decreased dose-dependent formation of 8-iso-$PGF_{2\alpha}$ and PGE_2 in irradiated HaCaT keratinocytes and supernatants (medium). The lowest prostanoid levels were observed with resveratrol and indole-3-carbinol. Generation of 8-iso-$PGF_{2\alpha}$ was very low in irradiated cells and medium. Microdialysate samples of all three treated areas showed reduced levels of F_2-isoprostanes and $9\alpha,11\alpha$-$PGF_{2\alpha}$ compared to UVB-irradiated areas, but higher levels than those of normal skin. Polyphenolic phytochemicals as oligomeric procyanidins, resveratrol, indole-3-carbinol, extracts from blueberry, pomegranate and ginkgo biloba seem to be potential inhibitors of UVB induced prostaglandin and isoprostane formation *in vitro* as well as oligomeric procyanidins, resveratrol and blueberry extract *in vivo*.

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Higher oxidative DNA damage in lymphocytes from piglets intra-gastrically administered with ghrelin, a natural ligand for the GHS receptor

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Ghrelin is produced by stomach cells and is a potent regulator of food intake, energy expenditure and growth hormone secretion. Ghrelin exerts also potent anti-inflammatory effects by inhibition of the expression of proinflammatory cytokines. In this work we investigated the effect of ghrelin on the susceptibility of peripheral blood mononuclear cells to oxidative stressors generated during inflammation. Ghrelin was administered intra-gastrically to newborn piglets for 7 days. After treatment, mononuclear cells (MNCs) were isolated and exposed to different DNA damaging agents: X-radiation (dose 0–3 Gy), H_2O_2 (0–250 μM), NaOCl (0–500 μM) and t-butyl hydroperoxide (0–1000 μM). The extent of DNA damage was evaluated by alkaline comet assay. To investigate the influence of ghrelin on cells' ability to repair DNA damage, MNCs were irradiated with 2 Gy of X-radiation and disappearance of DNA breaks was monitored 0.5, 1 and 2 h after irradiation. We found that ghrelin had a marked effect on the level of X-radiation-, H_2O_2- and SIN-1-induced DNA damage. In all cases, we observed a significant increase in the level of DNA strand breaks in ghrelin treated animals as compared with untreated controls. However, no effect of ghrelin was observed on cells' capacity to repair DNA damage. Our data point to a dual role of ghrelin in inflammation. On the one hand, it is known to inhibit the expression of pro-inflammatory cytokines and to have a potent anti-inflammatory action; on the other hand, intra-gastric administration of ghrelin potentiates the genotoxicity of oxidative stressors generated during inflammation.

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***Fucus vesiculosus* extracts in the prevention of atherosclerosis in apoE-deficient mice**

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The atherosclerosis is a multi-factorial disease that involves internalization of monocytes to the sub-endothelial space. Atherosclerosis can be modulated through diet and flavonoids and carotenoids can reduce the risk to suffer it. Thus, we addressed the question of whether aqueous and an ethanolic *Fucus vesiculosus* extracts, rich in phloroglucinol (polyphenol) and phloroglucinol and fucoxanthin (carotenoid), respectively, prevent atherosclerosis through modulation of endothelial adhesion molecules that favour the atheroma plaque formation. Male apoE-deficient mice (spontaneously develop atherosclerosis) were used. They were fed with diets containing 200 mg/kg body weight of either aqueous or ethanolic extracts of the seaweed, just after weaning and for 12 weeks. We measured at the aortic root and at the thoracic aorta the formation of the atheroma plaque by image analysis. Endothelial adhesion molecules expression was evaluated by immunofluorescence at the aortic root level. The aqueous extract reduced the stenosis ($p < 0.05$) and intrusion ($p < 0.05$) in the aortic root and these above lesions appeared before the ones in thoracic aorta. As a result, an important protective effect was also observed for both extracts at the thoracic aorta. The extracts reduced the expression of ICAM-1 by 30% and P-selectin by 35%, without modifying VCAM-1 expression. It is likely that the reduction of atheroma plaque induced by the seaweed extracts is related to the decreased endothelial adhesion molecules expression.

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Endogenously produced nitric oxide regulates oxygen consumption in rat hippocampal slices

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Nitric oxide ($^{\bullet}NO$) is an ubiquitous inter-cellular messenger molecule that plays essential roles in the modulation of vascular tone, neurotransmission and in the immune system. A current paradigm establishes that the binding of $^{\bullet}NO$ to cythochrome c oxidase in competition with oxygen results in the regulation of cellular respiration. We had previously shown that NMDA-evoked $^{\bullet}NO$ production is dramatically different among the principal cell layers of the hippocampal slices, with the following gradiente CA1 >> CA3 = DG. Considering the role played by $^{\bullet}NO$ in LTP on the CA1 sub-region and this regions higher sensitivity in several degenerative disorders associated with oxidative stress, we questioned whether NMDA evoked $^{\bullet}NO$ production in CA1 could effect tissue oxygen consumption. Using two microelectodes inserted into de CA1 st. Pyr. we succeed in simultaneously recording $^{\bullet}NO$ concentration dynamics and the evolution of pO_2. We observed that endogenously produced $^{\bullet}NO$ inhibited tissue oxygen consumption, with a direct correlation between extent of the inhibition of O_2 and the concentration of NO resulting from transient stimulation of the tissue with NMDA ($r = 0.78$). Also, the reversibility of the pO_2 profile seems to be determined by NO concentration: although for very low concentrations of $^{\bullet}NO$ (~ 30 nM) the inhibition observed is reversible, for higher concentrations of $^{\bullet}NO$, O_2 consumption was irreversibly inhibited. Both $^{\bullet}NO$ production and consequent O_2 consumption inhibition were dependent on calcium. These results obtained in a system close to the *in vivo* strongly support the current paradigm obtained in simple systems for oxygen and $^{\bullet}NO$ inter-play in the regulation of cellular respiration.

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***In vivo* alterations of cardiac mitochondrial function by the pro-oxidant anti-neoplastic agent doxorubicin: Role of the adenine nucleotide translocator**

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Doxorubicin (DOX) is a highly effective treatment for several forms of cancer. However, DOX induces a cumulative and dose dependent cardiomyopathy that has been ascribed to oxidative stress due to redox-cycling of the molecule on the mitochondrial complex I. Mitochondrial dysfunction, including induction of the mitochondrial permeability transition (MPT) and inhibition of mitochondrial respiration, have been implicated as major determinants in the pathogenesis of DOX cardiotoxicity. The adenine nucleotide translocator (ANT) has been suggested to be a principal component of the MPT pore and a possible target for DOX-induced cardiotoxicity. Nonetheless, no definitive evidence has been presented showing that altered ANT activity is due to a decreased amount of the protein. Evidence was found for decreased vitamin E content in heart mitochondria from treated animals simultaneous with increased sensitivity to diamide-induced MPT. Altered respiration in DOX-treated animals was reversed by both dithiothreitol and cyclosporin-A, which suggests that the inhibition of respiration results from thiol oxidation and increased MPT. By using

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carboxyatractyloside as a specific modulator of ANT activity and Western blotting, we also observed that following DOX treatment in rats: (1) the amount of 'functional ANT' that contributes to cardiac mitochondrial respiration with different substrates is reduced, (2) titrations with carboxyatracyloside revealed a lower threshold for MPT induction and, most importantly, (3) a specific decrease in the amount of the ANT protein. This study identifies the ANT as one important target for DOX-induced cardiac toxicity and correlates the decrease in ANT protein concentration with inhibition of mitochondrial respiration and increased ability to form or at least regulate MPT pores.

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Morphological hallmarks of doxorubicin toxicity on H9c2 myoblasts: Identifying new targets of a pro-oxidant anti-neoplastic molecule

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Doxorubicin (Dox) is a very potent anti-neoplastic agent used against several types of cancer, despite a cumulative cardiomyopathy that reduces the therapeutic window for treatment. The pro-oxidant action of Dox on cardiac cells has been proposed as the major mechanism responsible for the cardiotoxicity. When exposed to Dox, cells suffer not only biochemical alterations, but also morphologic changes. H9c2 myoblast cells have been extensively used as an *in vitro* model to study biochemical alterations induced by Dox treatment on cardiomyocyte cells. Despite the extensive work already published, there are few data available regarding morphological alterations of H9c2 cells during Dox treatment. The purpose of the present work was to evaluate Dox-induced morphological alterations in H9c2 myoblasts, focusing especially on the nuclei, mitochondria and cytoskeleton. The present work shows that, for the low concentrations, Dox causes cytoskeletal alterations including changes in nuclear lamina and cardiac myosin organization, mitochondrial depolarization and fragmentation, membrane blebbing with heterogeneous cell shape changes and phosphatidylserine externalization. For higher Dox concentrations, more profound alterations are evident, including nuclear swelling with disruption of nuclear membrane structure, mitochondrial swelling and extensive cytoplasm vacuolization. The results obtained indicate that Dox causes mitochondrial, nuclear and cytoskeletal morphological alterations in H9c2 cells, which are dependent on the drug concentration. Data obtained with the present study allow for a better characterization of the effects of Dox on H9c2 myoblasts, which have been typically used as a model to study Dox-induced cardiotoxicity, indicating new and previously unknown targets that can contribute to the selective cardiotoxicity of Dox.

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Biochemical rationale for the use of unsaponifiable fraction of olive oil as functional component of cosmeceutical producs for skin protection from environmental stressors

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Extra-virgin olive oil and its main unsaponifiable component, the triterpenoid unsaturated lipid squalene, are common ingredients of dermo-cosmetological preparations for the prevention/treatment of skin damage and photoprotection. Squalene (SQ) is also an important component (12%) of sebum lipids, unique to human skin and absent in all other primates. Our previous works have demonstrated its key role as sacrificial antioxidant, acting as a sensor on the skin of UV and other physical/chemical/microbiological environmental stressors. In olive oil, SQ is responsible, sinergically with tocopherols and polyphenols, for the long shelf life and resistance to elevated temperatures. The chemical composition of olive oil is interestingly similar to that of human sebum: with unusually elevated SQ concentrations and prevalence of mono-unsaturated and saturated fatty acids, the olive oil model provides relevant information on the physio-pathology of the skin. The possible role of SQ and sebum lipid oxidation in cell signalling and in the modulation of inflammatory mediators and cytokine network in the epidermal layer is speculated, with the consequent application of unsaponifiable fraction of extra-virgin olive oil, selectively enriched in polyphenols, topically on the skin, in combination with systemic supplementation of lipophilic natural antioxidants with skin trophism (RRR-alpha-tocopherol, Coenzyme Q_{10}), to guarantee the skin physiological balance between antioxidant defenses and the pro-inflammatory free radicals. In order to select parameters of quality of extra-virgin olive oil, able to provide efficacy of topical application on the skin function, we studied 41 different cultivars of olives from various geographical areas of Italy, to detect quali-quantitative differences in composition, focused on the unsaponifiable fraction, i.e. content of tocopherols, squalene, phytosterols and phenolic components, prone to oxidative decomposition. Analyses were performed by thin layer chromatography, spectrophotometry, gas-chromatography-mass spectrometry and high performance liquid chromatography. Differences were demonstrated on a geographical base (climate and soil composition) and in the course of the physiological cycle of fruit maturation. Levels of polyunsaturated linoleic and linolenic acid decreased (from 40.01% to 12.70% in the mature drupe), along with the increase of protecting levels of mono-unsaturated fatty acids, SQ increased (0.06% in the ripe drupe vs 3.20% in the harvested olives). Significant levels of verbascoside were also detected in the phenolic fraction, partly accounting for the strong antioxidant, anti-inflammatory and anti-microbial action of extra-virgin olive oil. The combined topical action of verbascoside, natural isomer of alpha-tocopherol and squalene on the skin, may account for the demonstrated efficacy of olive oil-based functional cosmeceutics in the protection from exogenous stressors and in the treatment of cutaneous chronic inflammation conditions with relevant social impact, such as acne.

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Smoking and oxidative stress expression in haemodialysis patients

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Oxidative stress (OS) in haemodialysis (HD) patients is characterized by the dysbalance in pro-oxidative–antioxidative activity. Considering the fact that in the population of HD patients there are many active smokers, we set the following goals: (1) to examine the influence of smoking on the changes of oxidative and anti-oxidative activity in HD patients; and (2) to compare the effects of active and passive smoking upon the OS expression in the same population. The study includes 111 patients of both sexes averagely aged 56.23 ± 11.84 on chronic HD–biocompatible treatment. The patients are divided into three groups: non-smokers ($n = 45$), active smokers ($n = 36$) and passive smokers ($n = 30$). In order to assess the oxidative activity we measured the serum xanthine oxidase (XO) and, as an indicator of the lipid peroxidation process, we measured malondialdehyde (MDA). The antioxidative activity markers were the values of serum catalase (CAT), glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) in erythrocytes. The results have shown that the active smokers on HD treatment had significantly higher OX values (89.45 ± 13.7), MDA values (17.97 ± 6.18) and GSH-Px (18.18 ± 4.85) (showed the greatest activity decline) in active smokers in comparison with the non-smokers OX (66.63 ± 10.12; $p < 0.01$), MDA (15.44 ± 5.20; $p < 0.05$) and GSH-Px (32.59 ± 9.44; $p < 0.001$) while there was no statistically significant difference in OX activity between the active and passive smokers (78.06 ± 11.93), MDA (16.82 ± 4.62) and GSH-Px (26.55 ± 7.39; $p < 0.005$). CAT and SOD values were also lowered in active smokers (CAT: 10.9 ± 2.87; SOD

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212.89 ± 39.74), but with no statistically significant difference with regard to the non-smokers (CAT: 11.68 ± 2.90; SOD: 219.29 ± 47.30) and the passive smokers (CAT: 11.92 ± 2.45; SOD: 208.77 ± 31.95). In conclusion, active, as well as passive smoking, is one of the factors that directly stimulates oxidative activity, leading to the oxidative stress expression in HD patients.

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Role of NADPH oxidase and calcium in the plant response to cadmium toxicity

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In plants, cadmium induces oxidative stress, although the mechanisms involved are complex and not well known. In this work, using *Arabidopsis* mutants deficient in NADPH oxidase (*Artboh-DF*), the cell response to this metal was studied and the role of calcium in the plant response was also investigated. Wild type plants (WT) and *Arabidopsis* mutants (*DF*) were grown in full-nutrient media supplemented with 50 μM $CdCl_2$ for 7 days and with or without 10 mM $Ca(NO_3)_2$. Cadmium produced a growth reduction in both WT and *DF* mutants, which was partially restored by the addition of calcium. In WT plants, Cd produced $O_2^{\bullet -}$ accumulation mainly in mesophyll cells, which was reverted by Ca^{2+}, while in *DF* mutants $O_2^{\bullet -}$ production was reduced. CuZn-SOD was down-regulated by Cd in both WT and *DF* mutants and was prevented by Ca^{2+} in WT plants. The expression of the heat shock protein, HSP15.7, was up-regulated by Cd in WT plants and Ca^{2+} reverted it. These results suggest that Cd-dependent $O_2^{\bullet -}$ accumulation is mainly due to a plasma membrane NADPH oxidase and $O_2^{\bullet -}$ is involved in the regulation of CuZn-SOD and HSP15.7. The Ca^{2+} deficiency induced by cadmium could affect the plant response to this heavy-metal.

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Protective effect *of eucalyptus globulus*, *juglans regia* and *hypericum androsaemum* leaf extracts against reactive nitrogen species and superoxide radical

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The plants *Eucalyptus globulus*, *Juglans regia* and *Hypericum androsaemum* have been used in traditional medicine for the treatment of several diseases such as asthma, skin inflammations and hepatic disorders. Considering that these diseases have been associated with oxidative and nitrosative stress, the aim of the present study was to evaluate the scavenging activity of superoxide radical ($O_2^{\bullet -}$), nitric oxide ($^{\bullet}NO$) and peroxynitrite anion ($ONOO^-$) by leaf extracts obtained from these plants. $O_2^{\bullet -}$ was generated by the NADH/phenazine methosulphate/O_2 system and the $O_2^{\bullet -}$ scavenging activity was determined by monitoring the effect of the leaf extracts on the $O_2^{\bullet -}$-induced reduction of nitroblue tetrazolium chloride at 560 nm for 2 min. The $^{\bullet}NO$ scavenging activity was measured by monitoring the effect of the leaf extracts on the oxidation of the non-fluorescent 4,5-diaminofluorescein to the fluorescent triazolofluorescein by $^{\bullet}NO$. The $ONOO^-$ scavenging activity was evaluated by monitoring the effect of the leaf extracts on the oxidation of the non-fluorescent dihydrorhodamine 123 to the fluorescent rhodamine 123 by $ONOO^-$. All the tested extracts exhibited scavenging activity against the studied reactive species. IC_{50}s (mean ± SE) of *E. globulus*, *J. regia* and *H. androsaemum* for $O_2^{\bullet -}$ were 8.39 ± 0.44, 47.59 ± 4.56 and 32.72 ± 3.44 μg/mL, respectively; for $^{\bullet}NO$ were 3.37 ± 0.15, 1.95 ± 0.29 and 2.18 ± 0.20 μg/mL, respectively, and for $ONOO^-$ were 1.28 ± 0.06, 1.66 ± 0.10 and 1.21 ± 0.09 μg/mL, respectively. Considering the well studied role of the mentioned reactive species in several pathophysiological conditions, as mentioned above, the observed antioxidant activity may account for the use of these plants in the prevention and/or treatment of oxidative stress-related diseases.

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Delayed aging through damage protection by the Arf/p53 pathway: Importance of antioxidant defence

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The Arf/p53 tumor suppressor module plays a central role in the detection and elimination of cellular damage, and this is at the basis of its potent cancer protection activity. Similar to cancer, aging is also associated to the accumulation of damage and, therefore, we have reasoned that Arf/p53 could have anti-aging activity by alleviating the load of age-associated damage. We have found that genetically manipulated mice with increased, but otherwise normally regulated, levels of Arf and p53 present a significantly delayed aging. This delayed aging is associated to a lower rate of accumulation of damage, notably including oxidative damage. These observations extend the protective role of Arf/p53 to aging, revealing a novel anti-aging mechanism and providing a rationale for the coevolution of cancer resistance and longevity.

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Preservatives induce oxidative stress, mitochondrial injury and P2X7 cell death recepteur activation: Application to Contact lens Multipurpose solution

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Purpose: Preservatives are known to induce severe side effects in dermatology, cosmetology and particularly in ophthalmology (main ocular toxic products). Contact lens multipurpose solutions, containing preservatives, are proposed to be rinse solution. Is rinse possible with preservatives containing solution? The aim of this work was to investigate the role of multipurpose solutions used to disinfect contact lens and the role of benzalkonium chloride preservative in P2X7 receptor-induced apoptosis on ocular surface cells.
Methods: Human conjunctival cells were incubated with multipurpose solutions and benzalkonium chloride concentrations. These solutions were compared to NaCl 0,9% and a ionized marine solution (Lacrymer). Microplate cytofluorometry, inverted fluorescence microscopy and flow cytometry were performed to evaluate cell viability (neutral red test), reactive oxygen species (ROS), superoxide anion production (dichlorofluorescein diacetate and hydroethidium tests), apoptosis via P2X7 cell death purinoreceptor activation (YO-PRO-1 test) and DNA fragmentation (sub G1 peak detection).
Results: Benzalkonium chloride was found to be the most cytotoxic preservative: as soon as 15 minutes of incubation, it induced necrosis with ROS overproduction. Even at very low concentrations, multipurpose solutions containing PHMB (biguanide preservative) or polyquad preservatives were found to be apoptotic with mitochondria-dependent oxidative stress. Ionized marine solution can decrease P2X7 cell death basal activation.
Conclusions: Benzalkonium chloride is used to preserve eye drops; when the active principle already presents antibacterial activity (such as fluoroquinolone), preservative should be avoided. Multipurpose solutions can induce side effects (dry eye for example) because of their preservatives that are cytotoxic. Contact lens should be rinsed with an inert solution (i.e: marine solution) after disinfection step.

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