2nd Spanish and Portuguese Congress on Free Radicals

November 25-27, 2004, Leioa-Bizkaia

A Joint Meeting of

Universidad del País Vaco / Euskal Herriko Unibertsitatea

Departamento de Fisiología
Fisiologi Saila

Facultad de Medicina y Odontología
Medikuntz eta Odontologi Fakultatea

GRUPO ESPAÑOL DE RADICALES LIBRES

Sociedade Portuguesa de Bioquímica

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IRALMET
Instituto de Investigación en Radicales Libres y Metabolismo

ABSTRACT BOOK

2ND SPANISH AND PORTUGUESE CONGRESS ON FREE RADICALS

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A Joint Meeting of

DEPARTAMENTO DE FISIOLOGÍA, UPV/FISIOLOGIA SAILA, EHU
GRUPO ESPAÑOL DE RADICALES LIBRES
SOCIEDADE PORTUGUESA DE BIOQUÍMICA
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Christian Scifo
(Catania, Italy)

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LECTURES

Opening Lecture

Free radicals in action

BOVERIS A

Laboratory of Free Radical Research, School of Pharmacy and Biochemistry, University of Buenos Aires, Argentina.

Free radicals in biological systems was a disputed subject from Gerschman's proposal of oxygen free radicals as the common mechanism of oxygen and radiation toxicity in 1954 to the discovery of superoxide dismutase by McCord & Fridovich in 1969. Recognition of the biological existence of molecules with unpaired electrons and high reactivity, implying a potential harmful role, led to an extraordinary activity in biomedical research and to the definition of the concept of oxidative stress (Sies, 1985) as an unbalance between oxidants and antioxidants that produces damage to biomolecules and cells. Nowadays, there are about 100 diseases, syndromes and pathological situations in which the concept of oxidative stress and oxidative damage are currently applied, which is accompanied by an active interest in the treatment or prevention of the mentioned diseases with antioxidant vitamins (vitamins E and C). Mitochondria are recognized as the subcellular organelles that are at the same time the most important source and target of free radicals. The continuous mitochondrial production of two primary free radicals (superoxide and nitric oxide) is currently investigated as the cause of pathological situations and of normal aging. Loss of neurological function and neurodegeneration are currently investigated as due to the cumulative effect of mitochondrial free radicals and to an unbalance of mitochondrial signaling. Nitric oxide and hydrogen peroxide are considered as normal metabolites that exert their signalling function in an optimal range of steady state concentrations, which if exceeded leads to a deleterious range and to cytotoxicity.

Round Table

Micronutrient deficiencies and toxic overload in the 21st century

MILLER NJ

Biolab Medical Unit, 9 Weymouth Street, London W1W 6DB, England

Investigation of "chronically unwell" patients usually reveals deficiencies of superoxide dismutase, glutathione peroxidase, zinc, magnesium and B vitamins, together with blocks in omega-6 and omega-3 essential fatty acids pathways. A mixture of toxic metals can be identified in blood and urine. Analysis of fat biopsies reveals accumulation of pesticides and other xenobiotics which have a short half life in the blood. These subjects may have accumulated toxins to concentrations below those of recognized toxicity, but the hepatic detoxification of such a wide variety of xenobiotics gives rise to sustained levels of free radical metabolites. Redox-active transition metals (such as mercury) also contribute to the development of "oxidative stress" states. Dietary micronutrient deficiencies (which are widespread and often unrecognized) thus create the conditions in which such disorders can develop.

Plenary Lecture I

Gene regulatory functions of vitamin E as deduced from metabolism

LANDES N, BUMKE-VOGT C, FLOHÈ L[+], HALLIGHAN E*, KARAKOULA A*, KLUTH D, MÜLLER-SCHMEHL K, PFLUGER P, SZTAJER H[#], LUNEC J*, BRIGELIUS-FLOHÉ R

*German Institute of Human Nutrition, Potsdam-Rehbrücke, Germany. [+]MOLISA GmbH, Magdeburg, Germany. *Dept Cancer and Molecular Medicine, Leicester University, UK. [#]Technical University of Braunschweig, Germany.*

Vitamin E is an essential micronutrient involved in various processes relevant to human health and disease. Whereas it has for long been considered just as an antioxidant, it now becomes clear that vitamin E has functions by far exceeding an antioxidative one. these include regulation of cellular signaling processes and gene expression. We learnt about these function from the findings that vitamin E is not inert but is metabolized and degraded. All forms of vitamin E are metabolized by side chain degradation initiated by an ω-hydroxylation catalyzed by a cytochrome P450 enzyme (CYP). This mechanism is identical for all forms of vitamin E, the degree by which individual forms are degraded, however, varies dramatically. CYPs degrade various endogenous and exogenous compounds and many of them are induced by their substrates via the activation of the pregnane-X-receptor (PXR). Also vitamin E identified as substrate of CYPs induced a reporter gene driven by PXR. The induction was highest with α- and γ-tocotrienol (T3) and low but significant with α-tocopherol. In addition, γ-T3 increased the mRNA of endogenous CYP3A4 in HepG2 cells (1). This shows that vitamin E is able to directly influence gene activity in principle which may explain many of the novel functions described for vitamin E in the last decade. To test whether the activity of other genes can be regulated by vitamin E, we performed genechip microarrays of liver RNA of mice grown with low, normal, and high amounts of α-tocopherol for three months. In addition, half of each group was supplemented with 250µg γ-T3/day for 7 days. Apart from Cyp3a11, the murine homolog of human CYP3A4, a specific set of α-tocopherol sensitive genes were identified which mainly were associated with cellular trafficking, exocytosis and calcium signaling. In contrast to the *in vitro* findings, CYP3a11 was not up-regulated by additional γ-T3. This can easily be explained by the high metabolism that is evident from the urinary excretion of γ-CEHC, the final degradation product of γ-T3 (2). The

different metabolic rate by which different forms of vitamin E are eliminated will definitely influence their biological activity.

1. Landes N, Pfluger P, Kluth D, Birringer M, Rühl ., Böl G-F, Glatt H, Brigelius-Flohé R (2003) Biochem Pharmacol 65, 269-273
2. Kluth D, Landes N, Pfluger P, Müller-Schmehl K, Weiss K, Bumke-Vogt C, Ristow M, Brigelius-Flohé R (Submitted) Modulation of CYP3a11 expression by α-tocopherol but not γ-tocotrienol in mice.

Lecture I

Physiology and molecular biology of the antioxidant effects of melatonin

RODRÍGUEZ C

Departamento de Morfología y Biología Celular. Universidad de Oviedo, Spain.

Studies on effects of the neurohormone melatonin have long focused on its regulation of circadian rhythms and reproduction. Melatonin, however, is present in living organisms as primitive as unicellular algae, where its hormonal status seems not necessary to synchronize any cellular rhythm. During the last decades several other interesting effects have been described for this hormone, such as its antiproliferative and antitumoral potential or its neuroprotective role. It has also been reported that melatonin possesses antioxidant properties: Antioxidant effects have been shown both at low and high concentrations. At low concentrations (physiological levels in plasma) melatonin regulates antioxidant enzyme activity and expression. At high concentrations it acts as a free radical scavenger, therefore preventing reactive oxygen species (ROS) damage on proteins, lipids and nucleic acids. Although clues on a link between these antioxidant properties along with the neuroprotective effect do exist, these have not been definitively proven yet. It has been shown how melatonin is able to regulate oxidative stress-dependent transcription factors in some neuroprotection models; also reported was an increase on antioxidant enzyme gene expression when melatonin protects dopaminergic neurons from oxidative stress induced by neurotoxins. Nevertheless, the intracellular pathway driving to both, inhibition of oxidative stress-dependent transcription factors and the increase in gene expression of antioxidant enzymes has not been elucidated. The first effect -that it occurs at high concentrations of melatonin- could be the consequence of its free radical scavenging properties, but we still continue work to confirm this and try to find out where in the intracellular signaling pathways melatonin acts. The second one occurs at low concentrations of the neurohormone, and it is likely due to either direct or indirect genomic effects of this hormone. The link between antioxidant effects and antiproliferative properties is a relatively new research area. Recent reports propose that oxidative stress increases cell proliferation while antioxidants tend to inhibits it. Melatonin inhibition of proliferation occurs in our experimental models at high concentrations, coincident with a decrease in the intracellular oxidative status of the cells and inhibition of oxidative stress-dependent transcription factors. Studies are on their way in order to elucidate the pathway step affected.

Lecture II

Redox activity of phenolic antioxidants and the potential benefits for human health

LARANJINHA J, FREITAS V*, GAGO B

*Faculty of Pharmacy and Center for Neurosciences and Cell Biology, University of Coimbra. *Dept. Chemistry, Faculty of Sciences, University of Porto, Portugal*

Evidence for the role of phenolic compounds present in human diet in the prevention of degenerative and cardiovascular diseases has been accumulating during recent years. Beyond its bioavailability, the multiple redox interactions of food phenolics with biological compounds are critical issues determining their bioactivity with consequences for health. This notion will be illustrated with phenolic acids and polyphenols from red wine encompassing: 1) its interaction with alfa-tocopherol/alfa-tocopheroxyl radical in micellar models with significance for the protection of low density lipoproteins against oxidation; 2) its reaction with nitrite producing nitric oxide ($^{\cdot}NO$). Concerning the former, phenols are reductants of alfa-tocoferoxyl radical in a structure-dependent fashion; among the wine flavanols tested, procyanidin dimer B2 gallate extracted from grape seeds exhibited very high reduction activity with consequences for LDL protection from oxidation. Regarding the later point, phenolic antioxidants may exert health benefits in the gasterointestinal tract. Saliva contains high amounts of nitrate and nitrite that suffer reduction by food phenolics, also present in saliva, yielding $^{\cdot}NO$. This reaction is favored by lowering the pH from 5.5 (often found in ischemia-reperfusion injury) to 1.5 (gastric acidity). Such non-enzymatic production of $^{\cdot}NO$ is discussed in connection with the health-promoting effects of plant phenols.

(Supported by FCT and FEDER, POCTI/AGR/42418/2001)

Plenary Lecture II

Role of oxidants and antioxidants in maintenance of muscle homeostasis

JACKSON MJ

School of Clinical Sciences, University of Liverpool, Liverpool, UK

It has been recognised for a considerable period that contracting skeletal muscle generates increased amounts of reactive oxygen and nitrogen species during exercise, but any potential physiological functions or adverse effects of these substances have been obscure. Recent data has provided substantial clarification of the nature of the reactive oxygen and nitrogen species generated by skeletal muscle at rest and during contractile activity and of the mechanisms of their generation. Surprisingly, the nature of the contractions undertaken by skeletal muscle influences both the level and the pattern of reactive oxygen and nitrogen species produced. Much discussion has centred on whether these species are key mediators of contraction-induced damage to skeletal muscle, but there is little firm data in support of this possibility. In contrast, reactive oxygen and nitrogen species are known to act to stimulate changes in signalling pathways and transcription factors that can influence gene expression and some new data support the possibility that they play this type of role in contraction-induced adaptations of skeletal muscle. These data have also prompted a reappraised of the potential roles of antioxidants in maintenance of muscle homeostasis although firm data for either positive or negative effects are lacking.

Our work is supported by the Wellcome Trust, BBSRC and United States National Institutes on Health.

Lecture III

Oxidative stress associated to different types of physical exercise. Effect of natural and synthetic antioxidants

GÓMEZ-CABRERA MC, DOMÉNECH E, BORRÁS C , SASTRE J, VIÑA J, PALLARDÓ FV

Departamento de Fisiología, Facultad de Medicina de Valencia.(SPAIN)

Exercise causes an increase in the generation of free radicals by cells. Exhaustive exercise generates excessive amounts of oxidative free radicals, that overwhelm cellular antioxidant defences and may cause tissue damage. We showed that these radicals cause cellular damage only when exercise is exhaustive. Strenous exercise causes oxidation of glutathione, release of cytosolic enzymes, and other signs of cell damage. They may, however, constitute signals to regulate muscle cell function. Accordingly, there is considerable interest in the potential of these mediators to regulate muscle adaptation to exercise. Moderate exercise increases lifespan in humans but this may be due to other healthy habits of the exercising population.

Xanthine oxidase is an enzyme involved in oxidant production and muscle damage during exhaustive physical exercise. Recent studies show that there is a redox regulation of cellular signalling and that the generation of reactive oxygen species leads to the activation of MAP-kinase pathway. This pathway induces the activation of the redox-sensitive transcription factor NF-kappaB which plays an important role in the regulation of gene activity.

The aim of this presentation is to review the role of the free radicals generated in exhaustive and moderate physical exercise, studying the expression of antioxidant genes and of transcription factors for mitochondrial biogenesis.

Our results show that exhaustive exercise causes activation of MAP kinases and of NFkB which is due to free radicals formed in exercise. The inhibition of xanthine oxidase with allopurinol prevents ERK ½, p38 phosporylation and NF-kappaB activation, expression of Mn-SOD, iNOS and eNOS in gastrocnemius muscle of rats after running until exhaustion.

Moderate exercise up-regulated the expression of antioxidant enzymes associated with longevity, such as Mn-SOD and GPx. We also found that moderate exercise up-regulated the expression of NRF-1 that is a key transcriptional activator of nuclear genes encoding mitochondrial enzymes and

Tfam, which stimulates mitochondrial DNA transcription and replication. However, supplementation with vitamin C or allopurinol during training prevented all of these adaptations.

We conclude that the usual practice of recommending antioxidant supplements before exercise should be seriously questioned. Oral antioxidant supplementation is very likely to be useful before competition when exercise is likely to be exhaustive, and damaging, but not when training.

Plenary Lecture III

Biomarkers of Oxidative Stress in Neurodegenerative Disorders

OLIVEIRA CR

Faculty of Medicine and Center for Neuroscience and Cell Biology, University of Coimbra, Portugal

Oxidative stress and reactive oxygen species have been shown to play a crucial role in the pathophysiology of neurodegenerative disorders, such as Parkinson's and Alzheimer's disease, amyotrophic lateral sclerosis and also in stroke.

Neuronal degeneration in Alzheimer's disease (AD) is associated with the deposition of dense-core plaques, which contain aggregated fibrillary β-amyloid protein (Aβ).

Cell culture data have shown that Aβ can induce an elevation of intercellular free calcium concentrations and of reactive oxygen species in neurons. These oxidative actions of Aβ increase the vulnerability of neurons to insults related to the pathogenesis of AD including metabolic impairment and excitotoxicity.

Mitochondria play a key role in the maintenance of cell energy and generation of free radicals, being also involved in cell death pathways, namely apoptosis. In a previous study we have demonstrated a reduced cytochrome c oxidase activity in AD platelets. Studies with cybrid cell lines also demonstrated that deficits in cytochome c oxidase in AD platelets could be transfered to ρ^o cells (cells depleted of mitochondrial DNA and functional mitochondria). In these cybrid cells, resulting from the fusion of ρ^o cells with platelets, an increase in free radicals production was shown to occur. In addition, when these cell lines were exposed to Aβ a depolarization of mitochondrial membrane, an increase in cytopalsmic cytochrome levels and caspase 3 activity were observed. Altogether these results suggest that mitochondria dysfunction is a relevant event occurring in AD neuronal cell death.

To determine whether peripheral biomarkers of oxidative stress are useful clinical indices of oxidation insults occurring in AD, three subject groups were recruited: Mild Cognitive Impairment (MCI), Mild AD and cognitively health controls. In these groups peripheral markers of oxidative stress were evaluated.

A significant activation of the glutathione cycle in AD and MCI patients could be demonstrated. Nevertheless, in these patients, decreased levels of antioxidant defences and increased levels of lipid and protein oxidation biomarkers were found. The results show that the complex pathology in AD is reflected in a pattern of altered serum concentrations of several marker molecules related to mechanisms thought to be of relevance for the pathogenesis of AD.

Research work supported by F.C.T.

Lecture IV

Gas chromatographic-mass spectrometric determination of F_2-isoprostanes: Application to *in vitro* and *in vivo* studies

WISWEDEL I

Otto-von-Guericke University, Medical Faculty, Department of Pathological Biochemistry, Magdeburg, Germany

F_2–Isoprostanes (F_2–IsoPs) are prostaglandin-like compounds generated by a non-enzymatic free-radical mediated oxidation of membrane-bound arachidonic acid. They are shown to represent reliable parameters of oxidative stress in various clinical and experimental conditions. F_2–IsoPs are characteristic in structure (cis-stereochemistry), ubiquitous in occurrence and stable *in* and *ex vivo*. They are not only biomarkers of oxidative stress, but also have biological effects; e.g. 8-iso-$PGF_{2\alpha}$, a major F_2–IsoP evokes vasoconstriction in lung and kidney. A large amount of work has been done in the field of isoprostane analyses. Methodologies described in the literature differ in the sample preparation steps or in the detection technique or both. Two main analytical approaches have been adopted: the first one involves immunological methods such as radioimmunoassays or enzyme immunoassays, which are easy to perform, but suffer from a lack of specificity as well as from potential interferences and/or cross-reactivities. The second approach is based on chromatographic separation and detection by mass spectrometry. Gas chromatography/mass spectrometry in the negative-ion chemical ionisation mode have been shown to be the most sensitive and precise quantitative method in the field of prostanoid research. This technique has been introduced and applied for the measurement of isoprostanes in different tissues (brain homogenates and mitochondria, cultured keratinocytes) and body fluids (mainly plasma samples and microdialysates of human skin), in various models of oxidative stress (iron/ascorbate, UVB-irradiation), in supplementation studies (flavanol-rich cocoa drink) and in different human diseases (chronic renal failure, psoriasis). Results from several experimental and clinical studies will be presented indicating the validity of isoprostanes as sensitive indicators of oxidant stress.

Lecture V

Maillard reaction-derived products in tissue proteins: new products and new perspectives

PAMPLONA R

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Non-enzymatic modifications of proteins can arise from direct exposure to reactive oxygen, chlorine, or nitrogen species generating oxidative products such as glutamic and aminoadipic semialdehydes, or from reaction with low molecular weight reactive carbonyl compounds derived from amino acids, carbohydrates or polyunsaturated fatty acids such as glyoxal, glycolaldehyde, methylglyoxal, malondialdehyde and hydroxynonenal, among other. These carbonyl compounds could react primarily with lysine, arginine and cysteine residues, leading to formation of both adducts (Advanced Glycation/Lipoxidation Endproducts, AGEs/ALEs) and cross-links in protein. Examples include the formation of CML, CEL, MDAL, argpyrimidine and lysine-lysine cross-links. Less reactive carbonyl compounds, such as glucose and other reducing sugars, can also react with proteins forming intermediate Amadori adducts that may evolve to form stable AGEs, such as pentosidine and CML. Reactive carbonyl species are formed in a variety of metabolic reactions. Some are generated by non-oxidative pathways such as the formation of methylglyoxal by the spontaneous decomposition of triose phosphates or during anaerobic metabolism of acetone and amino acids. Other carbonyl species derive from oxidative reactions. For example, glyoxal, methylglyoxal, and glycoaldehyde are formed during the autoxidation of carbohydrates. Lipid peroxidation reactions can also produce glyoxal and methylglyoxal. Carbonyl compounds dehydroascorbate, acrolein, and methylglyoxal are also produced during the oxidation of ascorbate, hydroxyamino acids, and polyunsaturated fatty acids, respectively.

In vivo, the relative significance of these different pathways of non-enzymatic protein modification would depend on the interplay between favouring factors such as the oxidative status and the concentration and reactivity of carbonyl species or their precursors, and the mechanisms of protection against damage mediated by the Maillard reaction through i) enzymatic inactivation, ii) the concentration of endogenous amines that can trap

carbonyl compounds, iii) the cellular removal and degradation of modified proteins and iv) the renal clearance.

Plenary Lecture IV

Nitric oxide, oxidants and protein tyrosine nitration: mechanisms and biological relevance

RADI R

Departamento de Bioquímica y Centro de Investigaciones Biomédicas, Facultad de Medicina, Universidad de la República, Montevideo, Uruguay

Protein tyrosine nitration under disease conditions represents a shift from the signal transducing physiological actions of ·NO to oxidative and potentially pathogenic pathways. Tyrosine nitration is mediated by reactive nitrogen species such as peroxynitrite anion ($ONOO^-$) and nitrogen dioxide ($\cdot NO_2$), formed as secondary products of ·NO metabolism in the presence of oxidants including superoxide radicals ($O_2\cdot^-$), hydrogen peroxide (H_2O_2) and transition metal centers. The precise interplay between ·NO and oxidants and the identification of the proximal intermediate(s) responsible for nitration *in vivo* have been under controversy but it is now clear that both peroxynitrite-dependent and independent pathways can contribute to tyrosine nitration *in vivo.* Typically, nitration pathways involve free radical biochemistry with carbonate radicals ($CO_3\cdot^-$) and/or oxo-metal complexes oxidizing tyrosine to tyrosyl radical followed by the diffusion-controlled reaction with $\cdot NO_2$ to yield 3-nitrotyrosine. While protein tyrosine nitration is a low yield process *in vivo*, 3-nitrotyrosine has been revealed as a relevant biomarker of ·NO-dependent oxidative stress; additionally, site-specific nitration "focussed" to particular protein tyrosines may result in modification of function such as the loss of activity of Mn-SOD and prostacyclin synthase or even a “gain of function” such as that observed in the case of cytochrome c and fibrinogen. Tissue distribution and quantitation of protein 3-nitrotyrosine, recognition of the predominant nitration pathways and individual identification of nitrated proteins in disease states open new avenues for the understanding and treatment of human pathologies.

Lecture VI

A ROS- and RNS-mediated role for peroxisomes in cell signaling

DEL RÍO LA, SANDALIO LM, CORPAS FJ, PALMA JM, ROMERO-PUERTAS MC, GÓMEZ M, BARROSO JB[1]

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In eukaryotic cells, peroxisomes are one of the main sites of intracellular H_2O_2 production, as a result of their oxidative type of metabolism [1]. Peroxisomes, like mitochondria and chloroplasts, can generate superoxide radicals ($O_2^{\cdot-}$), and have a complex battery of antioxidative enzymes, as well as different proteases [2]. In recent years, the presence of nitric oxide synthase (NOS) activity and the production of nitric oxide ($NO^{\cdot}$) has been demonstrated in plant peroxisomes [3,4].

In this communication, the metabolism of ROS and RNS in peroxisomes from senescent plants and from plants subjected to different types of abiotic stress, mainly including xenobiotics and heavy metals, will be analyzed. Results obtained suggest that peroxisomes can have an ambivalent role, as oxidative stress-generators and as a source of signal molecules, like $NO^{\cdot}$, $O_2^{\cdot-}$ and H_2O_2, in the signal transduction pathways of plant cells.

It seems reasonable to think that similar functions to these postulated for plant peroxisomes could also be performed by human, animal and yeast peroxisomes, where much less information is available on oxy radicals, antioxidants and nitric oxide.

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Plenary Lecture V

Mitochondrial function and regulation of cell signaling

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Mitochondria generate second messengers, such as H_2O_2 and $^{\cdot}NO$, which are involved in the regulation of redox-sensitive cell signaling through the mitogen-activated protein kinase (MAPK; *e.g.,* JNK) pathway, thus coordinating functional responses between mitochondria and other cellular processes. Conversely, mitochondria are the recipients of cytosolic signaling molecules, such as JNK, which translocates to mitochondria under stress conditions and during aging and elicits profound metabolic effects in the organelle through the activation of phosphorylation cascades.

Mitochondria are direct targets for JNK and aging increases translocation of phosphorylated JNK to mitochondria. The importance of mitochondria as a primary target for JNK-dependent apoptotic processes becomes increasingly apparent and is supported by the following observations: (*a*) JNK is a competent inducer of cytochrome *c* release from the intermembrane space of mitochondria, thus initiating an essential step in mitochondrion-dependent apoptosis in an imPT-independent manner and insensitive to cyclosporine A; (*b*) JNK mediates a partial collapse of $\Delta\psi m$, which is independent of imPT and cyclosporine A; (*c*) JNK phosphorylates Bcl-2/Bcl_{xL}, outer mitochondrial membrane-associated proteins that have been previously implicated in omPT. JNK also initiates a signaling cascade across both mitochondrial membranes resulting in phosphorylation of mitochondrial matrix pyruvate dehydrogenase and, consequentially, a decrease in its activity. The levels of mitochondrion-associated phosphorylated JNK increase with age and, thus, significant decreases of pyruvate dehydrogenase activity were observed in brain mitochondria as a function of age.

Mitochondria are major cellular sources of H_2O_2 and $^{\cdot}NO$. Mitochondrial H_2O_2 modulates the MAPK pathway activity, leading, for example, to activation of JNK upon dissociation of the glutathione transferase-JNK complex and/or suppression of phosphatases involved in JNK inactivation. $^{\cdot}NO$ generated by the mtNOS can differentially regulate the MAPK pathway by different mechanism. S-nitrosylation of JNK, for example, leads to its inactivation.

Impairment of the communication between mitochondrion-supported redox signaling and cytosolic signaling pathways may be the basis for the mechanisms inherent in cell death pathways and the loss of cell function associated with aging and age-related degenerative disorders. The interaction between these two processes establishes a regulatory device that controls cellular energy levels and redox environment.

Lecture VII

Role of oxidative stress in the apoptotic signaling induced by TGF-beta in hepatocytes

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Treatment of fetal rat hepatocytes with TGF-β is followed by apoptotic cell death. Using specific fluorescent probes by flow cytometry and confocal microscopy allowed us to probe that TGF-β mediates reactive oxygen species (ROS) production that precedes the loss of $\Delta\gamma_m$, the release of cytochrome c and the activation of caspases, all these effects being coincident with the decrease in the protein and mRNA levels of bcl-x_L. Presence of radical scavengers blocks the decrease in bcl-x_L levels, mitochondrial collapse and cell death, which indicates that during the apoptosis induced by TGF-β in hepatocytes, ROS mediate a mitochondrial-dependent cell death process. To determine the cellular source of the early ROS, we have used inhibitors that block different ROS-producing systems. Diphenyleneiodonium (DPI), which inhibits NADPH oxidase and other flavoproteins, completely blocked the increase in ROS induced by TGF-β, coincidently with an impairment of caspase-3 activation and cell death. Rotenone, an inhibitor of the NADH-dehydrogenase in mitochondrial complex I, attenuated, but not completely inhibited, ROS production, caspase activation and cell death mediated by TGF-β. No significant protection was observed with inhibitors of other ROS producing systems, such as cytochrome-P450 (metyrapone), cyclooxygenase (indomethacin) or xantine-oxidase (allopurinol). Additional experiments have indicated that two different mechanisms could be involved in the early ROS production by TGF-β. Firstly, an inducible (cycloheximide-inhibited) NADPH oxidase-like system could account for the extra-mitochondrial production of ROS. Secondly, TGF-β could increase ROS by a rapid down-regulation of antioxidant genes. Particularly, intra-mitochondrial ROS would increase by depletion of Mn-SOD. Finally, glutathione depletion is a late event and it would be more the consequence than the cause of ROS increase induced by TGF-β.

Lecture VIII

Sensing NO and cell viability by activated macrophages

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

Plenary Lecture VI

Sphingolipids: the virtuosity in apoptosis signaling

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Sphingolipids comprise a family of bioactive lipids that regulate diverse cellular functions such as cell proliferation, growth arrest or cell death. One of the best characterized members, ceramide can be generated in cells in response to various stimuli through various pathways including de novo synthesis or sphingomyelin hydrolysis by sphingomyelinases. Although several ceramide targets have been identified, one of the most relevant pathways whereby ceramide kills cells is through the interaction with mitochondria resulting in the apoptosome assembly. In addition, ceramide serves as the precursor for complex sialic acid-containing glycosphingolipids, including gangliosides, which are synthesized in the cytosol. In this regard, ganglioside GD3 mimics the ability of ceramide to target complex III of the mitochondrial respiratory chain, resulting in reactive oxygen species generation responsible for mitochondrial membrane permeabilization (MMP) and release of apoptogenic factors to the cytosol. Thus, in response to apoptotic stimuli, GD3 undergoes a substantial redistribution within the cell, disappearing gradually from the plasma membrane before its trafficking to mitochondria to elicit MMP. However, unlike ceramide, GD3 blunts the nuclear translocation of the transcription factor NF-κB, thus preventing the induction of antiapoptotic genes. Through this dual mechanism GD3 stands out as a versatile lipid death effector of relevance in cancer therary and pathophysiology.

Lecture IX

The effect of hydrogen peroxide in yeast metabolism

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When the yeast *Saccharomyces cerevisiae* is exposed to acute stress conditions, such as high cocentration of hydrogen peroxide, cells die or are growth arrested until they recovered to physiological conditions. To underlie the molecular mechanisms associated with cellular recovery of yeast cells after hydrogen peroxide stress, we analysed changes in gene expression at the genome-wide level. Genes encoding proteins involved in cell rescue and defence, redox homeostasis, protein folding and degradation, amino acid catabolism, nucleotide metabolism, transcription, ionic homeostasis, and cell wall and cytoskeleton organization were shown to be upregulated during cellular recovery. Most of the genes downregulated encode proteins involved in amino acid and protein biosynthesis. A significant number of upregulated genes (25%) were associated with proteolysis and amino acid catabolism. Indeed, the degradation of oxidised proteins in cells recovering from oxidative damage was partially dependent on the ubiquitin-26S proteasome.. This is clear from the results with cell deficient in *Doa4* ou *Pep4* genes, supporting the hypothesis that the main route involves the vesicular proteolysis. As a protein model we used glyceraldehyde-3-phosphate dehydrogenase and it was observed an increase of the turnover of this protein (Tdh) - a main target oxidatively inactivated during exposure to H_2O_2 - is associated with a decrease of Tdh carbonyl content. Concomitantly, Tdh activity was restored due to newly synthesised enzyme. These results provide evidence for the role of protein turnover after oxidative stress and highlight the changes in metabolic pathways leading to the recovery of cell physiological functions.

Plenary Lecture VII

Role of cholesterol in the progression of the atherosclerotic lesion

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Sustained high plasma cholesterol levels are recognized as able to increase the risk of developing atherosclerosis but the mechanisms by which cholesterol contributes to initiation and progression of atherosclerotic lesions are still subjects of debate. Cholesterol oxidation products (oxysterols) are consistently recovered in atherosclerotic plaques and may markedly enhance chronic inflammation, fibrosis and programmed cell death. Support to these statements also derives from investigation we performed on cells of the macrophage lineage challenged with 7-ketocholesterol (7K), unoxidized cholesterol or a biologically representative oxysterol mixture. Marked apoptosis was observed in macrophages when treated with 7K, while the same concentration of oxysterol mixture or unoxidised cholesterol did not stimulate programmed cell death. Further, we found that combined oxysterol treatment counteracted the ability of 7K given alone in up-regulating ROS steady-state level in macrophages by competing at the level of NADPH oxidase. The lack of direct toxicity again vascular cells by oxysterols in naturally occurring mixture may significantly facilitate their ability to modulate genes related to inflammation, which is the crucial driving force for fibrotic plaque towards progression. In this relation, by means of gene expression profiling, few genes encoding for chemokines, receptors, and adhesion molecules resulted to be over-expressed only when cells were treated with the oxysterol mixture. Once again, unoxidised cholesterol appears by far less reactive than oxysterols, pointing to oxidation as a crucial reaction to undergo for the sterol to exert pro-atherogenic effects.

Lecture X

Fish oil prevents atherosclerosis through modulation of oxidative stress

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Atherosclerosis is a complex multi-factorial disease and hypercholesterolemia decreases nitric oxide (NO) availability. Atherosclerosis can be modulated through diet and changes in cellular oxidative stress/antioxidant status. Fish consumption or fish oil (FO) supplementation reduces the risk of atherosclerosis (related to a reduction in serum lipid parameters, platelet aggregation and inflammation, and to an increase in HDL cholesterol) and improves endothelial function. We have found that a FO-rich diet increases NO-dependent relaxation in rat aorta. NO is essential for vascular tone regulation and hemodynamics, and prevention of LDL oxidation. Thus, we addressed the question of whether FO prevents atherosclerosis through NO production.

Healthy rats and apoE-deficient mice (prone to develop atherosclerosis) were fed diets containing 5% of either corn oil (rich in n-6 fatty acids) or FO (rich in n-3 fatty acids) just after weaning. Rats were fed for 8 weeks and mice were fed for different periods. At the end of the experiment we measured parameters related to oxidative stress, development of the atheroma plaque and cellular adhesion molecules.

In rats FO induced upregulation (31%) of mRNA endothelial NO synthase, responsible for the increase (90%) of NO in the aorta, without modifying the production of superoxide anion or enzymatic antioxidants. In mice the FO-rich diet increased NO release and reduced superoxide anion production, depending on the time of treatment. The lesions in the aortic valve sinus appeared before the lesions in aortic arch, and were reduced by FO in mice fed with normal or proatherogenic diets.

The increase in NO may restore the decrease of NO in high-risk situations at vascular level, and reduce LDL oxidation, (an early event in atherosclerosis). These results point to a new physiological mechanism of fish or FO in blood vessel-lipoprotein interactions, which may be important in the prevention/cure of vascular diseases.

Lecture XI

Biochemical and molecular mechanisms of oxidative stress in mononuclear cells of hypertensive subjects

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Recently, we have described the decrease of antioxidant enzyme activities and the increase of the oxidation products of lipids and DNA in whole blood and mononuclear cells of hypertensive patients. A positive correlation was observed between the values of MDA and both nuclear and mitochondrial 8-oxo-dG in the hypertensive cohort.

The objective of the present work was to assess the changes induced by antihypertensive treatment on oxidative status, antioxidant activities and reactive oxygen species byproducts in whole blood and mononuclear peripheral cells and to analyse the mRNA expression of most representative enzymes. Eighty-nine hypertensive patients (mean age 46, 46 men, average 24-hour blood pressure 139/88 mmHg, body mass index 29), were included. After three months of antihypertensive treatment (20 non-pharmacologic, 36 β-blockers, 33 angiotensin receptor blocker), oxidized/reduced glutathione ratio and malondialdehyde were significantly reduced, and the activity of superoxide dismutase, catalase and glutathione peroxidase was significantly increased in both whole blood and peripheral mononuclear cells. The content of damaged base 8-oxo-2'-deoxyguanosine in nuclear and mitochondrial DNA in hypertensive subjects was also significantly reduced during the antihypertensive treatment. In a subgroup of 42 subjects, the oxidative stress was further reduced and the antioxidant enzyme activities further increased after 12 months of antihypertensive treatment. The changes were independent of the kind of antihypertensive treatment.

We analyzed simultaneously the activity and the mRNA expression of the main antioxidant enzymes, SODs, CAT and GPxs and of those implicated in the GSH generation, glutathion synthase (GSS) and glutathion reductase (GRS) in hypertensive subjects in absence and during antihypertensive treatment.

The mRNA expression of the antioxidant enzymes and those which regulate the synthesis and regeneration of GSH were measured in controls and in hypertensives at baseline and at three months of antihypertensive treatment. mRNA expression of hypertensive subjects was significantly lower than that observed in cells from control subjects. The low levels in the hypertensives are more relevant considering the low enzymatic activity detected. The relationship between enzymatic activity and mRNA expression was established at lower levels in hypertensives than was observed in control subjects. After treatment, when enzymatic antioxidant activity increased, mRNA expression was further reduced.

In conclusion, antihypertensive treatment reduceses oxidative stress byproducts and increases the antioxidant levels of hypertensive subjects. This effect is independent of the kind of treatment and time-dependent. The lower activity of the antioxidant enzymes is accompanied by a reduction in the mRNA expression indicating a downregulation phenomenon. At the same level of enzymatic activity the mRNA was significantly lower in hypertensive patients that it was in the controls.

Plenary Lecture VIII

Aging related renal and vascular changes. The possible role of reactive oxygen species

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Aging is characterized by a progressively decreased glomerular filtration rate that seems to be the consequence of a slowly progressive renal scarring. Moreover, hypertension increases with aging. In order to analyse the mechanisms involved in the genesis of these changes, we studied renal aging in rats. In these animals, it was possible to demonstrate an increased synthesis of reactive oxygen species (ROS) and oxidative stress, with a simultaneous increase in the local expression of TGFß1 and extracellular matrix (ECM) proteins. The treatment with taurine prevented the changes observed in ECM accumulation in old rats. In telomerase deficient (TD) mice, it was also possible to demonstrate a progressive renal dysfunction and hypertension, which were related to an increased local oxidative stress. This oxidative stress was the consequence of disequilibrium between ROS synthesis and degradation, and was the direct consequence of telomerase deficiency and not telomere shortening, as it was evident in first-generation animals, in which the length of telomeres remained intact. The same oxidative unbalance was detected in mouse embryonic fibroblast (MEF) from TD mice. These animals showed increased values of blood pressure (BP) and a decreased hypertensive response after endothelin (ET) administration, with similar changes in BP after nitric oxide blockade. Circulating ET-1 and vascular ET-converting enzyme (ECE) expression increased in TD mice, suggesting ET-1 overproduction as the responsible for hypertension. To confirm these findings, studies were performed in cultured cells. Hydrogen peroxide (H_2O_2) induced an increased synthesis of TGFß1, with the subsequent increased extracellular matrix protein synthesis, in cultured mesangial cells. This effect seems to be dependent on the transcription factor AP-1. Moreover, (H_2O_2) increased the expression and the activity of ECE-1 in cultured endothelial cells. In summary, we propose that an increased local synthesis of ROS, perhaps as a consequence of a decreased telomerase activity, modifies the phenotypic expression of renal and vascular cells, with the subsequent renal and vascular dysfunction.

Lecture XII

Application of isoflavonoids on hypertension treatment

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Every day we have more evidences of the importance of the dietetic factors in the control of the arterial hypertension, this together with the high incidence of this pathology and the high cost of the pharmacological treatments, increases the interest for the antihypertension dietary measures. This way, besides the changes in the sodium and magnesium intake, it has been proved the positive action of the frequent and continuous administration of Vitamin C (superoxide anion scavenger) which is a good solution in a small percentage of hypertensions associated to the stress.

In the last years, several works showed the hypotensive effects of the administration of soy rich in isoflavones, in both, in rats and in human. Our group has recently contributed to this topic carrying out a randomized controlled trial in which, we gave a beverage intake with a high isoflavonoids content (120 mg of isoflavonoids were given every day, from them, 80 mg were genistein and 30 mg were daidzein), to 40 men and women with mild to moderate hypertension. The results shown significant decreases of about 12 mmHg in the mean blood pressure (1). From the obtained data we realize that it is necessary to ingest daily a minimum dose of 120 mg to get significant effects and that the hypotensive effects were detected in 55% of the patients. Moreover, the statistical study showed a high correlation between effect and isoflavonoids content, pointing to the genistein as the possible responsible hypotensive agent.

For us, there is enough information to think that isoflavonoids, mainly contents in the soy or together with their protein, are the main responsible for the hypotensive effects of a long term intake. They have many positive actions on the regulation of the arterial pressure. Among them, our group has described important salidiuretic effects like the inhibition of the Na-K-Cl cotransporter (NKCC2) (2), responsible for the renal recovery of the sodium in thick ascending limb with diuretic effect about a third of the furosemide (3). The elimination of sodium chloride helps to lose the excess of our diet. Other regulatory mechanisms of the arterial pressure have as target the diameter of the blood vessels and the isoflavones have also effects at this point, by relaxating

the arterial muscle as the results (4) found show, with a relaxing power similar to the furosemide but to dose 5 times lower.

Finally, when we study the free radicals scavenger actions of isoflavonoids we find an antiradical activity of middle strength (5) but with the advantage that its activity carries out in the whole organism and in the whole cells, for its capacity to go into the organic membranes.

The importance of the effect of isoflavonoids administration in the mild hypertensions is important because it represents around 80% of the total of hypertensive patients.

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ORAL COMMUNICATIONS

Oral Communication I

Cysteine-favanol conjugates as novel neuroprotective agents against oxytosis

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Neuronal damage associated with conditions such as Alzheimer's and particularly Parkinson's diseases has been related to oxidative processes in the early stages of cell degeneration. Using HT-22 nerve cells and glutamate-induced generation of mitochondrial reactive oxygen species (ROS) as a model for intracellular oxidative damage and the so called glutamate mediated programmed cell death or oxytosis, we found that inactive (–)-epicatechin (IC_{50}=610 μM) could be turned into effective (IC_{50}=35-65 μM) survival agents by derivatization with zwitterionic L-cysteine, its cationic ethyl ester derivative or its fully protected (*N*-acetyl-*O*-methyl) derivative. The conjugates were prepared by acid depolymerization of plant proanthocyanidins in the presence of the corresponding thiol. The slightly higher free radical scavenging capacity of the conjugates as compared with (–)-epicatechin could not account for the enhanced cell protection observed. Using dichlorofluorescein fluorescence and FACS analysis we found that the conjugates were able to scavenge intracellular ROS previously triggered by glutamate but again their scavenging power was too low to explain their survival promoting activity. The conjugates appear to exert their action mainly by interacting with the gluthatione metabolism. They were very effective at maintaining GSH levels in the presence of oxidative stress while (–)-epicatechin was not. The results indicate that modifications in the central ring of catechins may lead to new antioxidant compounds with neuroprotective activity by avoiding the depletion of endogenous antioxidant defences.

ROS-mediated enzymatic systems involved in the oxidative action of herbicide 2,4-dichlorophenoxyacetic acid

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2,4-Dichorophenoxyacetic acid (2,4-D) is an auxinic herbicide which, at low doses, is also used as a plant growth-promoting substance [1]. Chlorophenoxy herbicides are toxic for mammals and their ingestion can result in serious poisoning and fatal sequelae, but there is no consensus on a direct relationship between these xenobiotics and the physiological and metabolic dysfunctions attributed to them [2]. In this work, the effect of high 2,4-D concentrations on the ROS metabolism of pea plants was studied, in order to get some insights into the mechanism of action of this xenobiotic in plants.

In leaves from pea plants, 2,4-D increased the xanthine oxidase and superoxide dismutase activities, and induced the expression of the enzymes ascorbate peroxidase, monodehydro-ascorbate reductase, glutathione reductase, and catalase. 2,4-D also produced an increase in lipid peroxidation and protein oxidation, as well as an enhancement of the proteolytic activity.

The glutathione *S*-transferase system appeared to be involved in the detoxification of 2,4-D, and ROS-mediated enzymatic systems could be responsible for the overproduction of $O_2^{\cdot -}$ and H_2O_2 found in 2,4-D treated plants. In these conditions, oxidative stress can be induced, and this is probably accompanied by the degradation of proteins and other cell components. These results support the hypothesis that the mechanism of action of 2,4-D and other chlorophenoxy xenobiotics is possibly mediated by ROS, and this mechanism perhaps could also operate in mammals.

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Oral Communication III

Radical scavenging activity of new potential antioxidants based on natural molecules

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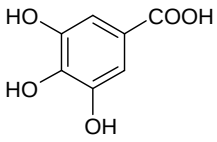
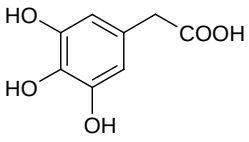
The design and development of new antioxidants is nowadays an emerging area of research since they could be useful either as therapeutic agents or as additives.

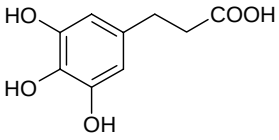
Being part of our work on antioxidants derived from cinnamic acids, new phenolic compounds were synthesized and their antioxidant profile evaluated through antiradical assays (ABTS, DPPH, 2'-dG, lipoperoxidation) in order to establish a structure - antioxidant activity relationship (SAR).



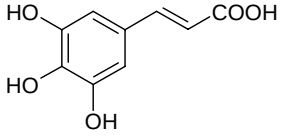
gallic acid



3,4,5-trihydroxyphenylacetic acid



3,4,5-trihydroxyhydrocinnamic acid



3,4,5-trihydroxycinnamic acid

The combination of natural antioxidants, in order to obtain an enhanced radical-scavenging activity while limiting the toxic response when applied to biological systems, should allow the rapid discovery of new interesting compounds.
The results of the SAR study will be presented.

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Oral Communication IV

Inhibition of skeletal muscle S1-myosin ATPase activity by the peroxinitrite-releasing agent SIN-1

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Impairment of muscle excitation-contraction has been reported upon exposure of muscle cells to chronic oxidative stress conditions, and it has been proposed that it is, at least in part, the result of oxidative modification of myofibril proteins [Mihm *et al.* (2001) *Circulation* 104:174-180; Canton *et al.* (2004) *Am. J. Physiol.* 286:H870-H877]. In this work, we have studied the sensitivity of S1-myosin (head segment of myosin responsible for the ATPase activity during muscle contraction) to the oxidative stress generated upon exposure to SIN-1, which slowly releases nitric oxide and superoxide anion and, as they rapidly react each other, slowly produce peroxynitrite. The results showed that exposure of S1 to SIN-1 produced a time-dependent inhibition of the F-Actin-stimulated Mg^{2+}-ATPase activity, reaching 50% inhibition with 46.7±8.3 mM SIN-1 for 8.7 mM S1. Differential scanning calorimetry of S1 (untreated and treated with different SIN-1 concentrations) pointed out that inhibition of S1 is paralleled by a protein modification that decreases the thermal stability of S1. In addition, SIN-1 also produced inhibition of the Ca^{2+}- and of the K^{+}-dependent ATPase activities of S1 with close IC_{50} values, thus, suggesting that the inhibition of F-Actin-stimulated S1 Mg^{2+}-ATPase activity is due to oxidation of the highly reactive Cys of S1, located close to the catalytic centre. This point was further confirmed by titration of S1 Cys with 5,5´-dithiobis-nitrobenzoic acid, and it was reinforced by the fact that the other common protein modification produced by peroxynitrite, e.g. nitrotyrosines formation, was less than 0.3 moles nitrotyrosines per mole of S1 at the concentrations of SIN-1 that produced nearly 90% inhibition of the F-actin stimulated Mg^{2+}-ATPase activity.

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Oral Communication V

Direct detection of singlet oxygen luminiscence in purple bacterial reaction center

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Singlet oxygen causes fast deterioration of photosynthetic reaction centers (RC's) type II under light stress conditions. It can be photogenerated at the heart of these RC's when triplet energy transfer takes place from the RC special pair consisting of (bacterio)chlorophyll ((B)Chl) to the surrounding molecular oxygen. The photochemical oxidation of (B)Chl's in RC's can be prevented by a carotenoid (Car) molecule that either accepts the (B)Chl triplet energy or quenches the photogenerated singlet oxygen. Though singlet oxygen is known to cause fatal inhibition of RC's, its photogeneration has been demonstrated mostly through indirect evidence (*e.g.* spin trapping EPR spectroscopy or fluorescence quenching of ROS sensors). The short lifetime of singlet oxygen in biological systems and the low sensitivity of commercial detectors measuring the near-infrarred luminiscence of singlet oxygen have hampered its direct detection in the past. In this work, we present for the first time the direct detection of singlet oxygen luminiscence in Car-less purple bacterial RC's from *Rhodobacter sphaeroides* R26.1, which in addition has lost, as a result of a specific chemical treatment, the first quinone acceptor (Q_A). The results presented here show not only the fast near-infrared luminescence decay of singlet oxygen, but also its rise. Under aerobic conditions, agreement between the decay of the triplet state of the special pair (P870) and the rise of singlet oxygen luminiscence points to P870 as the source of the photogenerated singlet oxygen.

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Doxorubicin induces ROS-mediated NF-κB signaling in cultured rat hepatocytes

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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 toxicity it produces, which has been ascribed to the continuos generation of reactive oxygen species (ROS) via a redox-cycling mechanism during the drug metabolization. We previously studied in primary cultures of rat hepatocytes the effects of DOX on the signaling pathway leading to the activation of NF-κB. Inmunocytochemical staining and Western blot analysis using specific anti-IκB-α- and anti-p65 antibodies revealed that DOX induced the cytosolic degradation of IκB-α and the p65 nuclear translocation in a dose- and time-dependent manner. In the present work, we have studied the NF-κB binding to its DNA consensus sequence and have analyzed the expression of Bcl-X_L and Bcl-2 mRNAs, which are known to be under NF-κB promoter control. The involvement of ROS in DOX-mediated NF-κB nuclear activation was also determined. Results showed that DOX dose-dependently stimulated p65 binding to the DNA consensus sequence. DOX increased both Bcl-X_L and Bcl-2 mRNAs levels. The cell-permeable SOD mimetic MnTBAP inhibited the translocation of p65 into the nucleus and the NF-κB transcriptional activation. The treatment of cells with DPI, which is known to inhibit flavin-containing enzymes, revealed similar results to those found for MnTBAP. Despite its ROS-mediated effects on NF-κB, DOX did not induce cell death or oxidative stress, expressed in terms of MDA and intracellular GSH. The present findings suggest a role for ROS, particularly O_2^-, in the translocation and transcriptional activation of NF-κB induced by DOX.

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Oral Communication VII

Transcriptional regulation of mitochondrial antioxidant defence system

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PGC-1α is a transcriptional coactivator that plays a major role in the regulation of oxidative metabolism, inducing mitochondrial activity and proliferation as well as oxidative lipid metabolism. We decided to investigate whether PGC-1α regulation of mitochondrial activity would also involve the regulation of genes that protect the mitochondria from oxidative stress and if so, whether this regulation might be physiologically relevant. We tested this idea in human vascular endothelial cells (HUVEC), where mitochondrial oxidative stress caused by hyperglycemia has been associated with endothelial dysfunction and cell death. Our results show that PGC-1α is present in vascular endothelial cells where its over-expression induces genes involved in mitochondrial activity, as well as genes that participate in the mitochondrial oxidative stress protective machinery. As a consequence of this induction a decrease in total cellular reactive oxygen species (ROS) levels was observed in cells that over-express PGC-1α. This reduction was particularly marked in the presence of inductors of mitochondrial oxidative stress such as high glucose or the mitochondrial electric transport chain blocker DMNQ. As a consequence of this protective effect, the drop in mitochondrial membrane potential that can normally be observed in cells exposed to high glucose did not occur in PGC-1α over-expressing cells. As mitochondrial dysfunction eventually leads to cell death, we analyzed PGC-1 ability to protect HUVEC cells from apoptotic cell death both under high glucose conditions and when treated with a high chemical dose of ROS, and found a strong reduction in apoptotic cell death associated with the presence of PGC-1α.

Therefore, we concluded that PGC-1 up-regulates the expression of genes that protect the mitochondria from oxidative stress. This modulation could help to prevent the patophysiological conditions that result from mitochondrial dysfunction caused by agents that increase mitochondrial ROS generation such as high glucose in diabetic patients.

Oral Communication VIII

PGE_1-dependent nitric oxide reduces D-galactosamine-induced cell death through attenuation of NF-κB activation and iNOS expression: *in vivo* and *in vitro* studies

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Introduction: Prostaglandin E_1 (PGE_1) reduces experimental liver injury. Nitric oxide (NO) may protect or exacerbate hepatocyte cell damage. We studied the role of NO during PGE_1 cytoprotection against cell death induced by D-galactosamine (D-GalN) in hepatocytes. **Material and Methods:** PGE_1 was pre-administered to D-GalN-treated male Wistar rats or cultured rat hepatocytes. NO production was regulated by iNOS inhibition or NO donor. The effect of TNF-α was assessed by anti-TNF-α antibodies. Different iNOS promoter regions fused to luciferase-reported gene including mutated or non mutated NF-κB sites were transfected to cultured hepatocytes. Apoptosis and necrosis were measured by DNA fragmentation and caspase-3 activation, and lactate dehydrogenase (LDH) release, respectively. NO end-products were measured by the Griess reaction. iNOS expression was assessed by WB and RT-PCR. NF-κB activation was determined by EMSA. **Results:** PGE_1-protection against D-GalN cell death was related to enhanced iNOS expression in rat liver, and this cytoprotective effect was prevented by iNOS inhibition. Anti-TNF-α abolished the effect of PGE_1 on iNOS expression and cytoprotection in D-GaN-treated rats. In vitro studies showed that PGE_1 enhanced NF-κB activation and iNOS expression in hepatocytes. But, PGE_1 reduced NF-κB activation, iNOS expression, NO production and apoptosis induced by D-GalN. L-NAME or NO donor reduced NO release and apoptosis by D-GalN. PGE_1 enhanced, and NO donor reduced iNOS promoter activity in transfected hepatocytes. **Conclusions:** 1) PGE_1 reduced D-GalN-induced cell death in liver and in cultured hepatocytes. 2) PGE_1 cytoprotection against D-GalN-induced apoptosis was related to the ability of the prostanoid to rapidly enhance NF-κB activation, iNOS expression and NO production in hepatocytes. 3) PGE_1-derived NO reduced NF-κB activation, iNOS expression, NO production and apoptosis induced by D-GalN in hepatocytes.

Oral Communication IX

Role of oxidative stress in the induction and post-translational modification of HSP25 following exercise in the liver

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This study was aimed at investigating the response to exercise of HSP25, a member of the family of small heat shock proteins (sHSPs), and to analyse the role of oxidative stress in its induction. The relevance of this response for the liver was suggested from experiments indicating that up-regulation of HSP25 was associated with an increased tolerance to hyperthermia, oxidative stress and TNF-α action. Analysis of ^{35}S-methionine incorporated in the charge variants of HSP25 in two-dimensional electrophoretograms of whole liver proteins, indicated that its rate of synthesis was reduced immediately after exercise, in clear contrast with what it happened to other inducible HSPs. A considerable proportion of the total radioactivity incorporated in HSP25 was found in a slightly more acidic variant ($HSP25_3$), the proportion of which reached a maximum when the total incorporation of radioactivity into the protein was minimal. Induction of HSP25 expression was confirmed by the abundance of its mRNA immediately after exercise, as compared with non-detectable amounts in liver samples of non-exercised animals. HSP25 accumulates transiently in the early post-exercise (approximately 10-fold the control values at 2 h post-exercise) followed by a reduction of its content that took place before the mRNA levels return to basal. This result contrasts with the reduced incorporation of radioactive methionine and suggests that the protein is freed out of liver cells early after exercise. The proportion of $HSP25_1$, the less acidic of the variants, increased immediately after exercise and was reduced thereafter when variants 2 and 3 grew, indicating early post-translational modifications. As previously reported for HSP72, HSP25 also showed two further waves of accumulation in the liver in the late post-exercise (8 and 48 h). Although during the early post-exercise, HSP25 levels did not correlate with free TBARS, they did with protein-bound TBARS ($\rho=0.637$, $p<0.001$) and HSP72 ($\rho=0.887$, $p<0.0001$) which suggests a connexion between oxidative stress an the induction and accumulation of HSP25 and other HSPs.

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Oral Communication X

Molecular mechanisms elicited by micronutrients in human prostate carcinoma cells

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Cancer, after heart diseases, is the second leading cause of deaths; in particular prostate carcinoma represents in men the most commonly diagnosed type of tumour and the second cause of death from cancer. It is characterised by a first early phase in which tumoral progression may be stopped/reduced by hormonal androgenic treatment and by a second very aggressive later phase in which clones hormone-refractory are present. In the present study the effects of two micronutrients were tested: resveratrol (200 μM)**,** and propolis ethanolic extract (100 μg/ml). The substances were added to androgen responsive (LNCaP) and androgen-resistant prostate cancer cells (DU145). A comparison with the activity of vinorelbine bitartrate (Navelbine®), a semi-synthetic drug of clinical use, was made. Several biochemical parameters were tested such as cell viability (MTT assay), cell membrane integrity (LDH release), cell redox status (nitric oxide formation, Reactive Oxygen Species production, reduced glutathione levels), genomic DNA fragmentation (COMET assay), presence of apoptotic DNA damage (TUNEL test) and mitochondrial transmembrane potential alteration ($\Delta\psi$). Morphological analysis (SEM and TEM) and immunoblotting evaluation of the expression of HSP27, HSP70, p21 and p53 have been also performed. Our results, together with providing a scheme of the possible molecular mechanisms activated, indicate the two micronutrients, either alone or in combination with Navelbine, as promising molecules for the therapy of prostate carcinoma, encouraging the development of *in vivo* studies aimed to check the actual feasibility of this kind of therapy.

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POSTERS

Poster - 1

SOD-enzymosomes: physico-chemical characterization and in vivo fate

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Superoxide Dismutase (SOD) may be used as a therapeutic agent for oxidative stress related diseases. However, its rapid elimination from the circulation is a major limitation. Therefore, strategies are currently being developed to improve the therapeutic action of SOD by increasing its plasma half-life, including the incorporation of the enzyme in delivery systems such as liposomes. Our strategy is to develop so-called SOD-enzymosomes by covalent attachment of the enzyme to the surface of long circulating liposomes, in order to achieve preferential accumulation at inflamed target sites, yielding therapeutic activity without the need for liposomal disruption. In this work, SOD was modified by acethylthioacetylation. The thiolated enzyme was coupled to the thiol-reactive linker [Maleimide (Polyethylene Glycol)2000] lipid. The SOD-enzymosomes obtained showed the following characteristics: mean diameter of 120 nm; high association efficiency (90%), good process yield (70%) and enzyme activity retention (60%). The *in vivo* fate of the SOD-enzymosomes was studied and compared with that of SOD not coupled to the surface but encapsulated in long circulating liposomes. Biodistribution and imaging studies were performed in rats injected i.v. with SOD-enzymosomes and liposomes labelled with ^{111}In-oxine. Hepatosplenic uptake was dominant for both formulations but the liver/blood and spleen/blood ratios were higher for the SOD-enzymosomes. The SOD-enzymosomes seemed to clear somewhat faster from the blood than the liposomes with the injected dose still present in the circulation at 24 h being 25% and 45%, respectively. We concluded that the SOD-enzymosomes present adequate characteristics and *in vivo* behaviour for use as a therapeutic agent.

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Poster - 2

The induction of foam cell formation by chylomicron remnants is promoted by antioxidants and depressed by oxidation of the particles

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The accumulation of lipid in macrophages forming foam cells is an early event in atheroclerosis. LDL is known to have a major role in the induction of foam cell formation, but oxidative modification of the particles is required for this effect. Recent work in our laboratory, however, has shown that chylomicron remnants (CMR), the lipoproteins which carry fats of dietary origin in the blood, cause extensive lipid accumulation in macrophages without prior oxidation. In this study, we have investigated the effects of the oxidative state of CMR on foam cell formation in macrophages derived from the human monocyte cell line THP-1, using oxidised particles and particles containing antioxidants. Chylomicron remnant-like particles (CRLPs), oxidised CRLPs (oxCRLPs), or CRLPs containing the antioxidants lycopene (lCRLPs) or probucol (pCRLPs) were incubated with THP-1 macrophages (48h) and the uptake and/or amount and type of lipid accumulated was determined. Uptake was assessed using CRLPs labelled with the fluorescent probe DiI and confocal microscopy or FACS. The lipid content of the cells exposed to oxCRLPs as compared to CRLPs was significantly lower, while that in those treated with lCRLPs or pCRLPs was substantially increased. These effects were mainly due to changes in the levels of triacylglycerol, although lCRLPs and pCRLPs also caused a significant increase in cellular cholesterol concentrations. Experiments with DiI-labelled CRLPs showed that oxCRLPs were taken up at a slower rate and pCRLPs at a faster rate than CRLPs. These results indicate that foam cell induction by chylomicron remnants, in marked contrast to LDL, is enhanced when they are protected from oxidation by incorporation of antioxidants, and depressed by oxidation of the particles. Thus, the protective effect of dietary antioxidants against atherosclerosis does not appear to be caused by inhibition of lipid uptake from CMR by macrophages in the artery wall.

Poster - 3

Effect of transcranial magnetic stimulation during 3-nitropropionic acid-induced oxidative stress in synaptosomes

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3-Nitropropionic acid (3NPA) induces an experimental model of Huntington's disease characterized by neuronal death and oxidative stress (OS). This study evaluates the effects of TMS on 3-NPA-induced oxidative stress in synaptosomes. Male Wistar rats were divided into five groups of six animals as follows: (i) Control, (ii) vehicle (iii) transcranial magnetic stimulation (TMS) (iv) 3-NPA, and (v) 3-NPA+TMS. 3NPA was administered i.p. at a dose of 20 mg/kg BW for 4 consecutive days in DMSO, whereas TMS was applied to 60 Hz and 0.7 mTesla daily for 8 days, beginning 4 days before and continuing for 4 days after the first injection of 3NPA. The OS induced by 3NPA was confirmed by a high level of lipid peroxidation products ($P<0.001$) in both striatum and whole brain synaptosomes, as well as an enhanced activity of superoxide dismutase ($P<0.001$). These changes were prevented by appplication of TMS. Moreover, TMS alone did not induce OS. In summury: (i) 3-NPA induces OS; and (ii) TMS decreases OS induced by 3-NPA. These data indicate the beneficial and neuroprotective effect of TMS against OS induced by 3-NPA.

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Poster - 4

Effect of sex steroids administration during 3-nitropropionic acid-induced oxidative stress in synaptosomes in ovariectomized rat

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Sex steroids play a role in the organization of brain development. 3-Nitropropionic acid (3NPA) induces an experimental model of Huntington's disease characterized by neuronal death and oxidative stress (OS). This study evaluates the effects of 17β-estradiol and testosterone (TEST) on 3NPA-induced oxidative stress in synaptosomes in ovariectomized (OVX) rat. Male Wistar rats were divided into nine groups of six animals as follows: (i) sham operated, (ii) 3-nitropropionic acid (3NPA), (iii) ovariectomy (OVX), (iv) 3NPA+OVX, (v) 3NPA+17β-estradiol (17bE), (vi) 3NPA+testosterone (TEST), (vii) OVX+17bE, (xii) OVX+TEST, (viii) 3NPA+OVX+17bE, (ix) 3NPA+OVX+TEST. 3NPA was administered i.p. at a dose of 20 mg/kg BW for 4 consecutive days in DMSO, whereas 17bE and TEST were injected s.c. at 0.6 mg/kg BW and 0.5 mg/kg BW, respectively. Sex hormones were administered daily for 8 days, beginning 4 days before and continuing for 4 days after the first injection of 3NPA. The animals were bilaterally ovariectomized according to the technique by Poumeau-Delille and under deep anesthesia (pentobarbital 40 mg/kg, i.p.). The OS induced by 3NPA was confirmed by a high level of lipid peroxidation products and protein carbonyls groups (P<0.001) in both striatum and brain cortex, as well as, an enhanced activity of superoxide dismutase (P<0.001) and a decrease of GSH content. Moreover, ovariectomy enhanced the OS induced by 3-NPA. These changes were prevented by previous administration of 17bE and intensified by pre-injection of TEST. Moreover, ovariectomy alone induced OS. In summary: These data indicate the beneficial effect of female sex hormone against OS induced by 3NPA and/or OVX in female Wistar rats.

Poster - 5

Effect of long-term dietary supplementation with thiolic antioxidants on the oxidative stress of peritoneal leukocytes from prematurely ageing mice

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According to the oxidative stress theory of aging, the oxidative damage induced by reactive oxygen species (ROS) generation is the most important fact underlying the age-related impairment of physiological functions. The balance oxidants/antioxidants is especially evident in the immune cells, that need to produce ROS to carry out their functions. We have characterized a model of premature ageing based on the different behavioural response in a simple T-maze test in which animals classified as prematurely ageing mice (PAM) show both a worse immune function and a shorter life span in comparison to non-prematurely ageing mice (NPAM). We have studied the effect of the two thiolic antioxidant compounds, N-acetylcysteine (NAC) and thioproline (TP) (0,1% w/w of each compound), ingested for 15 weeks on several parameters of oxidative stress in peritoneal leukocytes from middle-aged PAM and NPAM (12-month-old). The proinflammatory compounds (TNF-α, PGE_2 and NO), oxidant levels (oxidized glutathione) and peroxidative lipid damage evaluated as the level of malondialdehyde (MDA) are greater in leukocytes from PAM in comparison to those from NPAM. Further, antioxidant enzymatic defences (catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase) are lower in PAM. Diet supplementation with NAC and TP decreases the oxidant and inflammatory levels and increases the antioxidant enzymatic activity, especially in leukocytes from PAM. Therefore, a long-term dietary supplementation with thiolic antioxidants could be a useful strategy to prevent the oxidative stress of peritoneal leukocytes especially in aged subject such as the PAM.

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Poster - 6

Diet supplementation with antioxidants decreases oxidative stress in leucocytes from prematurely-ageing mice suffering lethal endotoxic shock

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Immune cells use ROS (reactive oxygen species) in order to support their functions and need adequate levels of antioxidant defences to prevent the harmful effect of an excessive ROS production. Since the levels of endogenous antioxidants decrease in oxidative stress situations, such as endotoxic shock or ageing, diet supplementation with antioxidants may be useful to prevent the age-related immune impairment and the negative effects of oxidative stress in sepsis. In the present study, we have analised oxidative parameters (oxidized glutathione, GSSG, ratio GSSG/GSH, tumor necrosis factor, TNF-α, prostaglandin E_2, PGE_2), antioxidant defences (reduced glutathione, GSH; catalase, CAT) and also lipid peroxidation (malondialdehyde, MDA), in peritoneal leucocytes from 12 month old prematurely aging mice (PAM) and non prematurely aging mice (NPAM). They received a diet supplemented with 20% (w/w) of biscuits enriched with antioxidants (vitamin C, vitamin E, β-carotene, zinc and selenium) for 20 weeks. Endotoxic shock was caused by intraperitoneal injection of E.coli lipopolysaccharide (LPS) (50mg/Kg). The samples were obtained at time 0 (before endotoxin injection) and at 2h and 24h after LPS administration. The endotoxemia increases the concentration of inflammatory and oxidant compounds and lipid peroxidation, as well as the GSH levels. However, the CAT activity is reduced. These effects are more pronounced in PAM with respect to those found in NPAM. The diet supplementation with antioxidant compounds decreases oxidative stress and reduces oxidative damage, and therefore increases the survival of supplemented PAM and NPAM 65% and 40%, respectively, in comparison with the control groups.

Poster – 7

Mice showing high levels of anxiety, and specially male, suffer an increased oxidative stress in heart and kidney cortex

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Anxiety involves an increased oxidative stress (higher content of oxidants and lower of antioxidants) in individuals suffering from it. We have characterized a model of premature ageing in mice based on the different behavioural response in a simple T-maze test in which animals showing a lower performance (classified as PAM or Prematurely Ageing Mice) exhibit high levels of anxiety, a decline in nervous and immune functions, and a shorter life span when compared to good performers (NPAM or Non-Prematurely Ageing Mice). Since ageing is associated with an oxidant/antioxidant imbalance, the shorter life span of males as compared to females is probably related to their higher oxidative stress. In the present work we have studied, on heart and kidney cortex from adult Swiss female and male NPAM and PAM, oxidative stress parameters, i.e.: oxidized (GSSG) and reduced (GSH) glutathione levels and peroxidative lipid damage, i.e.: malondialdehyde (MDA) levels. Results show an increased oxidative stress (higher GSSG and lower GSH levels) and greater MDA levels in PAM in comparison to NPAM, and in males with respect to females. There is a gradation in the oxidative stress level, being maximum in PAM males and minimum in NPAM females, which confirms the present model of anxiety. In addition, in general, there are no significant differences between NPAM males and PAM females, which suggests that the different behavioural response to emotional stress situations is as important as sex to determine the oxidative state of animals and consequently their longevity.

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Poster - 8

Xanthine oxidase increases in the heart of prematurely ageing mice

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Xanthine oxidoreductase (XOR) is known for its catalytic role in purine degradation, metabolizing hypoxanthine and xanthine to uric acid with concomitant generation of superoxide anion. XOR has two forms, xanthine oxidase (XO) and xanthine dehydrogenase, which transfer electrons from xanthine to oxygen and NAD^+, respectively, yielding reactive oxygen species (ROS) and NADH. XOR seems to be implicated in oxidative stress-related diseases such as endotoxemia and in oxidative stress-related processes such as ageing. In agreement with the most widely accepted theory proposed to explain ageing, the oxidation theory, free radicals and ROS are responsible for the impairement of cell functions through the oxidative damage to biomolecules, specially in fixed postmitotic cells. We have a model in mouse of premature ageing in which adult animals that not performe successfully in an exploratory test (a T-maze) show some immune and nervous characteristics of old animals, oxidative stress in their leucocytes and a shorter life span than mice performing succesfully in the test. The aim of this study was to investigate the activity of XOR in heart (an organ containing predominantly fixed postmitotic cells) of prematurely ageing mice (PAM) and of non prematurely ageing mice (NPAM) suffering an endotoxic shock produced by intraperitoneal injection of lypopolysaccharide (LPS, 50 mg/Kg), which causes the death of animals 30 h after LPS administration. The enzymatic acitivities were measured by spectrofotometry. The activity of total XOR as well as the percentage of XO were significantly higher in the heart of PAM than in that of NPAM. These results suggest that XOR could contribute to the oxidative condition found in ageing.

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Poster - 9

The antioxidant activity of galloylated catechins is related to highly ordered structures in membranes different than the cholesterol-induced L_o phases

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We have previously demonstrated a correlation between catechins antioxidant activity and the presence of highly ordered lipid structures, according to fluorescence anisotropy results and membrane solubilization assays. In this study, catechins with galloyl moiety, epicatechin gallate (ECG) and epigallocatechin gallate (EGCG), were more effective in scavenging AAPH-induced peroxyl radicals than non-galloylated catechins, epicatechin (EC) and catechin (C). This fact was correlated to the formation of ordered lipidic structures.

We have further characterized these lipid structures by fluorescence anisotropy studies using DPH, TMA-DPH and Laurdan probes, differential scanning calorimetry (DSC), bilayer transition to micelle studies and octadecyl rhodamine fluorescence self-quenching. The results indicated that galloylated catechins increased the amount of detergent required for the bilayer-to-micelle transition in a higher degree than cholesterol does, being this effect more intense in the gel phase. Fluorescence anisotropy measurements revealed that whereas EGCG decreased acyl chain mobility of the superficial part of the bilayer, ECG did it through all bilayer length. Furthermore, ECG was more effective than EGCG in increasing the self-quenching of octadecyl rhodamine, which indicates the presence of highly constricted structures in the membrane. The difference between ECG and EGCG capacity was attenuated when cholesterol was present at a molar ratio compatible with L_o phase formation. DSC studies showed that ECG decreased the interaction of lipid with cholesterol and formed catechin-enriched lipid structures. These results lead us to postulate that ECG induces ordered structures in biological membranes different than L_o phase. The modification of the physical properties of the membrane and the formation of ordered structures by galloylated catechins may be important to explain some aspects of the catechins biological activity such as its radical scavenging activity.

Poster - 10

Different stage of HCV liver disease is associated with a different pattern of lipoperoxidation

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Hepatitis C virus (HCV) infection exposes to the risk of chronic hepatitis with progression to cirrhosis and hepatocellular carcinoma. Two major hallmarks in HCV infection are liver steatosis and increased production of reactive oxygen species. It is common believing that the oxidative damage progresses with liver tissue injury, however whether and how the pathogenesis of HCV-steatosis is linked to oxidative damage is still debated. Aim of this study is to assess Low Density Lipoprotein (LDL)-peroxidation indexes in relation to the liver injury. LDL (1.006-1.063 g/ml) was isolated by sequential ultracentrifugation from plasma of 10 HCV patients with histological diagnosis of Chronic Hepatitis of slight entity (CH) and of 7 HCV-Cirrhotic patients (CI). Conjugated diene formation was assayed by measuring the absorbance at 234 nm. The end point content of aldehydic products of oxidation were assayed on LDL before (LPO) and after stimulation with Cu^{2+} (LPO-Cu). Principal component analysis of the results gave rise to two significant Principal Components (PC) explaining the 80% of total variability. PC1 scales with the Ratio LPO/LPO-Cu together with diene formation-rate (Pearson r = 0.91 and r = 0.80 respectively), while PC2 is dependent on both the LPO and LPO-Cu (Pearson r = 0.83 and r = 0.81 respectively). In the plane spanned by PC1 and PC2, CH and CI patients segregate showing a different pattern of LDL oxidation. In particular, CH and CI have comparable levels of LPO-Cu (9.9±7.9 and 9.1±10.9 nmol/μg triacylglycerol, respectively), while LPO content in LDL is significantly lower in CI than in CH (13.3±10.9 and 62.6±61.1 nmol/μg triacylglycerol, respectively; $p<0.05$). In conclusion, lipid oxidation is an early event in the HCV-related hepatitis, but does not correlate with liver injury. In cirrhotics liver functional impairment is prevalent on oxidation and probably changes occurring in LDL structure become more important.

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Poster - 11

Nitric oxide regulates PGC-1α expression in endothelial cells

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NO (nitric oxide) plays a major role in endothelial physiology. On one hand, it is known that NO can react with the active site of cytochrome C and cause the blockade of the electron transport chain (ETC). On the other hand, it has been proposed that NO is a positive regulator, in brown adipose tissue (BAT), of the mRNA levels of PGC-1α (peroxisome proliferator-activated receptor-γ coactivator 1α), a major regulator of lipid metabolism and mitochondrial biogenesis and activity. Experiments from our lab have shown that PGC-1α is present in vascular endothelium, where its overexpression not only promotes mitochondrial activity but also results in the induction of genes involved in mitochondrial oxidative stress protection. Because of the important role of PGC-1α and NO in cellular metabolism and oxidative stress protection, we decided to investigate the effect of NO on PGC-1α expression and its putative effects on PGC-1α mitochondrial targets (like cytochrome C). BAECs (bovine aortic endothelial cells) and HUVECs (human umbilical vein endothelial cells) were treated with the NO donors DETA-NO and SNAP, at different doses (15.5μM-496μM) and time points (1h-24h). Contrary to the expectation we observed, in all cases, a downregulation in the mRNA levels of PGC-1α and its target genes, while treatment with the nitric oxide sintase inhibitor L-NAME had the opposite effect. Then, we studied whether or not this negative effect was dependent on the activation of sGC (soluble guanilate cyclase). We found that, in cells treated with ODQ (a sGC inhibitor) the effect of NO donors on PGC-1α expression was reverted. Furthermore, treatment with 8-Br-cGMP (a membrane permeable analog of cyclic GMP), mimicked the effects of NO donors. These results show that NO is a negative modulator of PGC-1α expression in primary endothelial cells, and support the relevance of NO in the regulation of energy metabolism.

Poster - 12

High endogenous ascorbate content does not prevent water stress effects on pea nodule metabolism

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Legume nitrogen fixation (NF) is commonly described as a very sensitive process to a wide range of environmental stresses, being water shortage among them. Nodule sucrose synthase (SS) activity is inhibited under early water stress conditions and, furthermore, it is also inhibited by a range of different stresses. Thus, SS has been proposed as a key enzyme controlling nodule metabolism under stress conditions (1). Oxidative stress has been shown to occur in nodules under water stress (2). In well-irrigated soybean plants, exogenous ascorbate caused an increase in nitrogenase activity and a marked delay in nodule senescence (3). This study examines the hypothesis that increment of the endogenous ascorbate levels may alleviate the oxidative damage caused by free radicals produced under water stress and, therefore, prevent the decline of NF and the related response of nodule metabolism. A set of four weeks old pea plants were irrigated during 48 hours with the nutrient solution (4) containing 5 mM L-galactono-γ-lactone (GL), the natural ascorbate precursor. After this period, plants were subjected to drought by reducing irrigation from 100 to 3 ml day^{-1}. This treatment was compared to a set of well-irrigated and water-stressed plants, but omitting GL treatment in both cases. Ascorbate content increased significantly within the nodules of the GL treated plants compared to those of control plants. However, this higher content of antioxidant molecules did not alleviate the decline of NF and the down-regulation of SS provoked by water stress.

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Poster - 13

Protection against UV damage by antioxidant polyphenolic fractions from wine by-products

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Solar radiation in the UV range is the major source of adverse reactions in the skin and is one of the most efficient environmental carcinogen known. Since many harmful effects of UV radiation are associated with the generation of reactive oxygen species (ROS), antioxidants as polyphenols are a promising strategy to work against the oxidative damage induced by it.

We evaluated the free radical scavenging activity (DPPH assay) of different polyphenolic fractions, obtained from pressing de-stemmed Parellada grapes (*Vitis vinifera*), and demonstrated that all the fractions were more effective than the vitamin E analogue Trolox. Furthermore, we studied the possible capacity of the original fraction OW and other preparative chromatography-derived fractions to attenuate UVB-induced oxidative stress-mediated phosphorylation of MAPKs caused in quiescent human keratinocites HaCaT. The activation of ERK1/2 and p38 induced by UVB in HaCaT cells decreased when they were pretreated with fraction OW for 24h.

These findings prove that these polyphenolic fractions from wine by-products have the potential to inhibit UVB-induced oxidative stress, suggesting they may be useful for the prevention and treatment of a variety of solar UV light-induced human skin disorders.

Poster - 14

Tamoxifen prevents brain mitochondrial injury induced by oxidative stress-related events

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This study evaluated the effect of the synthetic, nonsteroidal antiestrogen drug tamoxifen on brain mitochondria function. We observed that tamoxifen concentrations above 30 nmol/mg protein induced a slight decrease on RCR and ADP/O ratio. However, only higher concentrations of tamoxifen (≥ 70 nmol/mg protein) affected the phosphorylative capacity of mitochondria. Those effects were characterized by a decrease on mitochondrial transmembrane potential ($\Delta\Psi m$) and repolarization level and an increase on repolarization lag phase with a decrease in ATP levels. Moreover, our results also show that tamoxifen presented a potent capacity to inhibit hydrogen peroxide formation and reduced the extent of lipid peroxidation induced by the pro-oxidant pair ADP/Fe^{2+}. Tamoxifen also exerted some protection against mitochondrial permeability transition pore (MPT) opening, although in a less extension than that promoted by cyclosporin A, the specific inhibitor of the MPT. However, in the presence of tamoxifen plus cyclosporin A, the protection observed was significantly higher when compared with that induced by both agents alone. Furthermore, tamoxifen avoided the oxidation of thiol groups and GSH depletion promoted by Ca^{2+}.

These results show that tamoxifen can afford protection against brain mitochondrial injury promoted by several oxidative stress-related events such as hydrogen peroxide production, lipid peroxidation and the induction of the MPT. Since numerous neurodegenerative diseases are intimately related with mitochondrial dysfunction, future therapeutical strategies could be designed taking in account this protective role of tamoxifen.

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Poster - 15

A distintic FeSOD protein in pea nodules

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The superoxide dismutases (SODs) are enzymes that catalyze the removal of O_2^- via a dismutation reaction. SODs in plants are classified into three major types based on their metal cofactor, which is either copper plus zinc (CuZnSOD), manganese (MnSOD) or iron (FeSOD). CuZnSODs are inhibited by KCN and H_2O_2, MnSODs are resistant to both and FeSOD are inhibited by H_2O_2 but resistant to KCN (Dalton, 1995). Plants contain all three types, CuZnSODs are present in the cytosol, nucleus and apoplast; the MnSODs in the mitochondria and peroxisomes; and the FeSODs in the plastids and cytosol (Moran et al, 2003). In pea plants two FeSODs isozymes located in the plastids has been described (Gómez et al. 2004). These FeSODs are inhibited by H_2O_2.

In this work, we show a strong FeSOD band in native gels of nodule protein extracts, which revealed resistance to H_2O_2. This protein was induced when plants were exposed to paraquat, herbicide which generates reactive oxygen species.

Two plastidic FeSODs can be detected in leafs by western blotting, while in nodules there is just one and expressed at a lower level. The report of this distintic FeSOD in pea nodules suggests the existence of at least two families of FeSODs within the same organism. Further studies are in progress to elucidate the function and localization of this new nodular FeSOD.

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Poster - 16

Western analysis of FeSOD from legumes

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Superoxide dismutases (SODs) are a family of metalloenzymes that catalyze the dismutation of superoxide anion radicals to molecular oxygen and hydrogen peroxide. Iron superoxide dismutases (FeSODs) are expressed only in plants. Plants FeSODs have always been described as chloroplastic enzymes. Recently, a new and highly active FeSOD with cytosolic localization has been reported in the plant *Vigna unguiculata* (cowpea), and two families of FeSOD have been described among nodules of legumes based on phylogenetic and immunological data (Moran et al, 2003).

In this work, we use the antibody against the cowpea FeSOD and a new antibody against pea chloroplastic FeSOD. This new antibody has been produced using as antigen a synthetic peptide based on a recent 3D model for cowpea FeSOD (Muñóz et al., 2003), and on the reported sequence for a pea chloroplastic isozyme (Moran et al., 2003). Western blots indicate the existence of the reported cowpea FeSOD, and two bigger FeSOD bands which response differently to abiotic stresses and to the age of the leaves.

In pea plants, the two antibodies detect two bands in SDS gels at a similar size to the previously reported (Gómez et al. 2004). A further analysis of pea plant FeSODs is present in a separate poster by Marino et al. The results obtained with the cowpea leaves support the idea that the two different families, cytosolic and plastidic, can be present among the same legume.

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Muñoz IG, Moran JF, Becana M, Montoya G (2003) Acta Crys: D59:1070
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Poster - 17

Aluminum-induced pro-oxidant effects in rat hippocampus. Gene expression of antioxidant enzymes after melatonin administration

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The aluminum (Al)-induced pro-oxidant activity, the protective role of exogenous melatonin, as well as the mRNA levels of some antioxidant enzymes were determined in the hippocampus of rats following administration of Al and/or melatonin. Two groups of male rats were intraperitoneally injected with Al (as Al lactate) or melatonin only, at doses of 7 and 10 mg/kg/day, respectively, for 11 weeks. During this period, a third group of animals received Al (7 mg/kg/day) plus melatonin (10 mg/kg/day). At the end of the treatment, hippocampus was removed and processed to examine the following oxidative stress markers: glutathione transferase (GST), reduced glutathione (GSH), oxidized glutathione (GSSG), superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx), catalase (CAT), thiobarbituric acid reactive substances (TBARS), as well as protein content. The gene expression of Cu-ZnSOD, MnSOD, GPx and CAT was evaluated by real-time RT-PCR. On the other hand, Al, Fe, Mn, Cu and Zn concentrations in hippocampus were also determined. The results show that Al exposure promotes oxidative stress in the rat hippocampus, with an increase in Al concentrations. The biochemical changes observed in this tissue indicate that Al acts as pro-oxidant agent, while melatonin exerts an antioxidant action by increasing the mRNA levels of the antioxidant enzymes evaluated. The protective effects of melatonin, together with its low toxicity, as well as its capacity of increasing mRNA levels of antioxidant enzymes, suggest that this hormone might be administered as a potential supplement in the treatment of neurological disorders in which oxidative stress is involved.

Poster - 18

Effect of melatonin on hepatotoxicity and oxidative damage induced by paracetamol (acetaminophen)

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Melatonin has been shown to be an effective antioxidant in a number of experimental models both *in vitro* and *in vivo*. The aim of this study was to evaluate melatonin protection from oxidative damage in the human liver cell line (HepG2), after incubation with paracetamol (APAP). We investigated the effect of melatonin treatment on several processes. These include cell viability, necrosis, apoptosis, lipid peroxidation and oxidative damage. We therefore investigated melatonin and APAP hepatotoxicity. Cells were exposed to melatonin (0.0001 - 5 mM) with or without APAP (0.1 - 80 mM) at 12, 24 and 48 h using the colorimetric MTT assay. Moreover HepG2 cells were treated with melatonin (1 mM) and APAP (40 mM) for 48 hr. Necrosis was assessed by LDH leakage. Oxidative damage was evaluated by measuring intracellular reduced and oxidized glutathione (GSH, GSSG), and glutathione peroxidase and reductase activities (GPx, GR). Apoptosis was investigated by flow cytometry and lipid peroxidation was determined by measuring the concentration of thiobarbituric acid reactive substances (TBARS). We found that: 1. Melatonin protects HepG2 cells from APAP-induced apoptosis, necrosis and lipid peroxidation; 2. Melatonin protects HepG2 cells from APAP-induced oxidative injury. The antioxidant effect of melatonin was revealed by an increased intracellular GSH level, associated to cell viability improvement. Moreover, GPx and GR activities were significantly increased.

Poster - 19

The role of the yeast neutral sphingomyelinase Isc1p in cell death associated with oxidative stress and ageing

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The *S. cerevisiae* ISC1 gene encodes a phospholipase C homologous to the mammalian neutral sphingomyelinase. Isc1p hydrolyses inositol phosphosphingolipids, releasing ceramide. In mammals, ceramide is an important regulatory component of differentiation, cell cycle arrest, stress responses, apoptosis and senescence. In this work, we analysed the role of Isc1p in oxidative stress resistance and cell ageing. Our data shows that the disruption of ISC1 gene decreased hydrogen peroxide stress resistance and chronological life-span of yeast cells. In both conditions, an increased accumulation of oxidised proteins and lipid peroxidation products was observed in isc1 mutants. However, the basal levels of major antioxidant defences or heat shock proteins, were not affected in isc1 mutants. In wild type cells, senescence was associated the oxidative inactivation of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (TDH) and glutathione depletion. Similar results were observed in the isc1 mutants. These results suggest that the oxidative stress sensitivity and the accelerated ageing of isc1 mutants did not result from a deficient antioxidant capacity. It was previously shown that the yeast Yca1p caspase mediates programmed cell death associated with oxidative stress and senescence. Oxidative stress-induced cell death in isc1 mutants was also an active process. In agreement, hydrogen peroxide stress sensitivity of isc1 mutants was suppressed by disruption of YCA1 gene. The results obtained indicate that sphingolipid signalling mediated by Isc1p plays a key role in cell survival during oxidative stress and ageing in yeast.

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Poster - 20

Novel flavan-3-ol conjugates with cysteine derivatives: Good candidates for potent and safe antioxidants

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New antioxidants resulting from the conjugation of flavan-3-ols with cysteine derivatives have been obtained by acid depolymerisation of grape polymeric procyanidins. The free radical scavenging studies showed that, in contrast with underivatised (–)-epicatechin, the new compounds were able to donate more hydrogen atoms than electrons to 1,1-diphenyl-2-picrylhydrazyl (DPPH, hydrogen donation) and tris(2,4,6-trichloro-3,5-dinitrophenyl)methyl (HNTTM, electron transfer) free radicals, respectively. Two compounds among those tested were found with interesting antiradical capacities. 4β-[*S*-(*O*-ethyl-cysteinyl)]catechin showed higher antiradical power against DPPH than its epicatechin counterpart and elevated H/e^- ratio. 4β-[*S*-(*N*-acetyl-*O*-methyl-cysteinyl)]epicatechin 3-*O*-gallate showed the highest hydrogen donating capacity (10 H per molecule) while keeping the electron transfer capacity low (2.9 e^- per molecule). It presented the highest H/e^- ratio among all the thio-conjugates tested so far. All the conjugates were growth inhibitors human colon carcinoma HT29 cells. Cell cycle and apoptosis studies have also been conducted. The same two compounds were found to be the most potent antiproliferative agents triggering little changes in cell cycle and low induction of apoptosis. A feature common to both compounds might have a positive influence on both their hydrogen donor capacity and antiproliferative potency.

Poster - 21

Protective role of insulin against neuronal metabolic dysfunction induced by oxidative stress

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It is widely known that insulin stimulates glucose uptake and metabolism in non-neuronal cells. Despite the scarce information about its role in neuronal cells, recent evidences suggest that insulin may have a neuroprotective role against oxidative stress, closely related with several neurodegenerative diseases, namely Alzheimer's disease. In previous studies we determined that insulin prevents lipid and protein oxidation induced by ascorbate/Fe^{2+} in rat cortical neurons. In this study we investigated the metabolic role of insulin upon oxidative stress conditions, induced by ascorbate/Fe^{2+}, in cultured cortical neurons. Insulin (0.1 or 10 μM) stimulated [^{3}H]-deoxyglucose uptake and prevented the decrease in pyruvate/lactate induced by oxidative stress, suggesting the stimulation of glycolysis. Moreover, insulin prevented the decrease in membrane integrity, determined by LDH leakage. Insulin also prevented oxidative stress-mediated decrease in intracellular ATP/ADP and the increase in extracellular ATP and adenosine. In order to determine whether the extracellular adenosine pool resulted from extracellular ATP degradation or occurred through the reversal of adenosine transporter, we have also tested the effect of AMPCP, an inhibitor of 5'-ectonucleotidase, and NBTI, an inhibitor of adenosine transporter. Upon oxidative stress, a major pool of extracellular adenosine was shown to result predominantly from ATP degradation. Our data suggest that the decrease in extracellular adenosine accumulation observed in the presence of insulin may underlie the neuroprotective role of this peptide against oxidative stress-mediated changes in neuronal metabolism.

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Poster - 22

Peroxidative effects exerted by *p*-hydroxybenzenediazonium ion in two in vitro biological systems

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The toxicity of arenediazonium ions is believed to result from the appearance of very reactive compounds during the dediazoniation process. In the case of the *p*-hydroxybenzenediazonium ion (PDQ), radical species generated during dediazoniation could potentially initiate lipid peroxidation. The data obtained in spectrophotometric and chromatographic experiments suggest that an interaction between PDQ and linoleic acid (LA) gives rise to the characteristic absorption of oxidized products deriving from LA. Moreover a chromatographic analysis of the decomposition of PDQ in the presence of 2-methylcyclohexa-2,5-diene-1-carboxylic acid (CHD) shows that aryl and peroxyl radicals abstract a hydrogen atom from CHD, in accordance with our general scheme for PDQ dediazoniation described in a previous publication. We confirm the existence of peroxidative effects derived from the PDQ dediazoniation in two *in vitro* systems: rat liver enriched membrane fractions P2 and rat intestinal epithelial cell line IEC-18. The results show that PDQ exerts toxic effects in cells as stated by the increase in LDH activity compared with untreated cells (355.0 ± 20.1 *vs* 3499.1 ± 137.3 $mU.mL^{-1}$; $P < 0.05$), and this effect is derived from an oxidative damage which is correlated with an increased production of MDA (6.3 ± 0.2 *vs* 54.1 ± 2.6 nmol mg $Prot^{-1}$; $P < 0.05$) and GSH depletion (5.4 ± 0.7 *vs* 0.4 ± 0.1 nmol mg $Prot^{-1}$; $P < 0.05$). In addition PDQ induced lipid peroxidation in enriched membrane fractions dose dependently.

Poster - 23

Quercetin inhibits cytokine and iNOS expression through the inhibition of NF-κB pathway

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Quercetin is a common antioxidant flavonoid, which has been proposed to exert different biological actions including anti-inflammatory, antihypertensive as well as antiproliferative in numerous cell lines. In the present study we have assessed the *in vitro* mechanisms involved in the anti-inflammatory effects of quercetin on macrophage biology, cells that play a crucial role in inflammation. For this purpose, murine bone marrow-derived macrophages (BMDM) were incubated in the presence of different concentrations of quercetin (1, 10, 50 μM) during 1 hour and then stimulated with LPS (100 ng/ml). The results revealed that quercetin reduced dose-dependently the expression (as detected by Western blotting) and secretion (by 40%) of the pro-inflammatory cytokines TNFα and IL-1β in LPS-stimulated BMDM. In addition, quercetin also reduced iNOS expression in these stimulated cells. When the transcription factor signaling pathways involved in the inhibitory effects showed by quercetin were studied, we observed by western blotting that the flavonoid inhibited in a dose-dependent manner the phosphorylation of the IκB-α, hence inhibiting the activation of the NF-κB pathway, but did not affect JNK activation. In conclusion, the antioxidant properties of quercetin can participate in the downregulation of pro-inflammatory cytokines and iNOS expression via inhibition of the NF-κB pathway, since this is a redox-sensitive transcription factor.

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Poster - 24

Antioxidant activity in the first stages of the melanin biosynthesis pathway

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The melanin biosynthesis pathway begins with the hydroxylation, with molecular oxygen, of L-tyrosine to L-dopa, catalysed by the enzyme tyrosinase. In turn, the tyrosinase oxidises, with molecular oxygen, L-dopa to *o*-dopaquinone, which, through a series of chemical reactions, regenerates L-dopa in the medium. The antioxidant capacity of monophenols (for example, L-tyrosine) is well known, as is the greater antioxidant power of *o*-diphenols (L-dopa). In this biosynthetic pathway, antioxidant capacity is generated (L-dopa), which, when it decreases, slows down and even stops the melanin biosynthesis pathway, since tyrosinase, to reach its steady state rate, acting on L-tyrosine, needs a minimum level of L-dopa in the medium. We have demonstrated that free radicals (tyrosyl) generated by ultraviolet light (λ=245nm) or by the peroxidase/hydrogen peroxide enzymatic system consume the L-dopa generated in this pathway. The capture of these tyrosyl free radicals involves the production of quinones (*o*-dopaquinone, dopachrome, *p*-topaquinone), resulting in an increase in the pro-oxidant capacity of the system, which are less toxic for the cell than the free radicals.

Poster - 25

Antioxidant activity of phenolic compounds from rosemary (*Rosmarinus officinalis* L.) and their effect on the membrane physical properties

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The antioxidant activity of rosemary extract is related to the presence of phenolic abietane diterpenes like carnosic acid (CA) and its derivatives carnosol (CAR) and rosmadial (RDAL), and also to the occurrence of other phenolic compounds such as flavonoids (genkwanina, GW) and phenolic acids, especially rosmarinic acid (RA). Since abietane diterpenoids and non-glycosilated flavonoids are highly hydrophobic, their antioxidant activity is expected to occur in a lipophilic environment such as cell membranes, where they can accumulate and protect against lipid peroxidation. Nevertheless, the presence of these molecules in the membrane also may affect the physical properties of the bilayer, as we have previously demonstrated for other phenolic compounds, which suggests a more complex and membrane-related mechanism underlying their biological activity.

In this work, the hydrophobicity of the major compounds deriving from rosemary (CAR, CA, RDAL, GW, RA) was estimated by determining the *n*-octanol/water partition coefficient (log $P_{o/w}$) and correlated to their effect on the fluidity of the bilayer. The antioxidant activity of these compounds and two rosemary extracts (one lipophilic and other hydrophilic) was also determined both in a free and in a liposome-based system by applying TEAC and TBARS assays, respectively. Steady state anisotropy measurements using fluorescent probes located at different depth in the membrane showed that the flavonol genkwanin considerably decreased the acyl chain mobility at all lengths of the bilayer, whereas the diterpenes CAR, CA and RDAL decreased the fluidity of the phospholipid palisade at an internal position. The results showed a correlation between the capacity of diterpenes to lower the mobility in deep regions of the bilayer and their antioxidant activity in a membrane model system.

Poster - 26

Development of a fluorescent biosensor for the detection of antioxidants

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A fluorescent method for the detection on antioxidant capacity is described, which is based on the superoxide anion measurement using a highly sensitive fluorescent biosensor recently developed in our laboratory [1]. The mechanism of the biosensor is based on the coupled reaction of the enzymes superoxide dismutase (SOD) and horseradish peroxidase (HRP), which are coimmobilized in a sol-gel matrix, together with the use of the probe N-acetyl-3,7-dihydroxyphenoxazine (Amplex Red). Within the biosensor, SOD catalyzes the dismutation reaction of the generated superoxide radical with the release of hydrogen peroxide, which, in the presence of HRP reacts stoichiometrically with the non-fluorescent Amplex Red to generate the red-fluorescent oxidation product resorufin.

As a first application of this biosensor, the superoxide scavenging properties of a series of herbal extracts and their oxidant components were investigated. Radical superoxide was generated from xanthine/xanthine oxidase system and from liposomes composed of unsaturated phospholipids. The response of the biosensor to a fix concentration of superoxide radical was determined in presence and in absence of different concentrations of antioxidants such as trolox, lauryl gallate and some tea catechins. The antioxidant capacity against superoxide radical generation was determined and compared to the total antioxidant capacity, measured with the traditional methods.

[1] Pastor et al. *Anal Biochem*. In press, 2004

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Poster - 27

Beneficial role of vitamins in lead-induced oxidative stress

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The potential role of oxidative stress injury, which is associated with lead poisoning suggest that antioxidants may enhance the efficacy of treatment designed to mitigate lead-induced toxicity. To investigate this hypothesis, female *Wistar* rats were exposed to 300 mg/L of lead, as drinking water, alone or in combination with some naturally occurring antioxidants (vitamins A, E, C and B_6) during pregnancy and lactation. Lipid peroxidation was estimated by thiobarbituric acid reaction (TBARS), according to the method of Okhawa et al (1979), and catalase (Aebi, 1983) and superoxide dismutase (Marklund and Marklund, 1974) activities were evaluated in the brain of pups at 1 and 21 days of age. A decrease statistically significant was observed in body and brain's weight of lead-exposed animals, but combined administration of lead and vitamins was able to increase pup's weight towards normal. The concentration of brain TBARS, a reflection of the endogenous level of lipid oxidation was significantly higher (+ 30%) in the Pb-treated group than in control group at both ages studied. Vitamins treatment reduced brain TBARS to control levels. Surprisingly, no significant changes were found in the activity of antioxidant enzymes studied in lead-treated group, although in lead + vitamins-group a significant decrease in catalase activity with respect to control group was noted.

From our findings, it appears that the simultaneous administration of lead and vitamins during gestation and lactation prevents some of the deleterious effects of lead, mainly in TBARS production.

Poster - 28

Cellular signaling involved in the production of nitric oxide by human endothelial cells

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Endothelial dysfunction is one of the first stages in the development of atherosclerosis, and endothelial nitric oxide synthase (eNOS) modifies the release of nitric oxide (NO). Our research group reported modulation of eNOS by flavonoids and fish oil in a rat model. We thought interesting to study some key points involved in the modulation of NO pathway by using human endothelial cells in culture. To this end, we have developed several techniques related to cell signalling and to a direct measurement of NO production as NO levels are very low even when cells are activated (of the order of pM-nM).

Human endothelial cells (EAhy926) at confluence were subjected to several treatments for 20 h 25 min: control, bradykinin (BK) (added in the last 15 min), phorbol 12-myristate-13-acetate (PMA) and PMA plus BK, all of them with or without N-monomethyl-L-arginine (L-NMMA), an inhibitor of eNOS, to optimise culture conditions. We measured the expression of PKC_{α} and eNOS by Western blot, the production of NO by fluorescent probes with 4,5 diaminofluorescein (DAF-2), and cGMP by immunoassay.

PKC_{α}, activated by PMA was involved in the expression of eNOS, which increased by 50%. Although PMA increased NO production by 500%, it did not affect cGMP. The production of NO and cGMP increased after the BK treatment, as BK activates eNOS, which in normal conditions is inhibited by caveolin-1. Incubating endothelial cells with PMA + BK had a synergistic effect on eNOS and NO.

In conclusion, we measured the NO released by endothelial cells in culture directly. NO production is supported by changes in other molecules involved in its generation.

We thank Dr. Cora-Jean S. Edgell from the University of North Caroline (USA) for providing the cell line.

Poster - 29

Serum ROS production and the pregnancy attainment in subfertility

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Oxidative stress, which is characterized by a high production of reactive oxygen species (ROS), may play a role in reproductive diseases. In the present work, we have studied the possible interactions between ROS production and the pregnancy attainment in women under an *in vitro* fertilization (IVF) program. During an IVF cycle patients undergo a strict hormonal treatment in order to produce the ovarian hyperstimulation before the embryo transfer. The plasma 17ß-estradiol levels change from undetectable values (E_2 min) to supraphysiological levels (E_2 max). The possible modifications induced by the IVF cycle on both the oxidation kinetic and the total antioxidant activity (TAA) of unfractionated serum were studied at two stages (E_2 min and E_2 max) during the cycle, and the variable changes (E_2 max - E_2 min) determined. Serum was oxidized by copper ions and the reactions were followed spectrophotometrically at 245 nm and 268 nm. The oxidation parameters were calculated from the oxidation curves. The total antioxidant activity was measured by the $ABTS^{\bullet+}$ decolorization method. The oocyte number obtained after one cycle was also determined. Results showed a significant negative correlation (R = - 0.42) between the number of oocytes and the variation of the time to attain the maximal oxidation rate (t_{Vmax}). The variations of both TAA and t_{Vmax} during the IVF cycle were significantly higher in women getting pregnant. These differences could be due to the different E_2 max levels reached during the hormonal treatment. These results suggest that the serum redox state influences pregnancy attainment.

This work was supported by MSC (FIS PI020233). Grants MCYT, Gobierno Vasco and UPV.

Poster - 30

Analysis by ARMS of the polymorphism in the targeting sequence of the MnSOD gene

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Manganese superoxide dismutase (MnSOD) is the primary antioxidant enzyme that protects cells from oxidative stress by catalyzing dismutation of superoxide ($O_2^{\bullet -}$) to hydrogen peroxide (H_2O_2) and molecular oxygen in the mitochondria of eukaryotic cells. The T1182C substitution at the second exon of the MnSOD gene changes valine (Val) to alanine (Ala) in the targeting sequence of the protein, this mutation being associated with a decreased defence capacity against oxidative stress. The aim of this work was to extend a new methodological approach to determine the Ala-9Val MnSOD polymorphism. To our knowledge, this is the first study reporting the characterization of the MnSOD allelic variants through a simple polymerase chain reaction without any further restriction enzyme digestion. We have used four different primers in the same master mix. The PCR renders fragments of 189 bp (Val) and/or 366 bp (Ala), depending on the presence of T or C allele. A 514 bp band is always obtained as control of the success of the amplification. We have applied this amplification refractory mutation system to describe the genotype distribution of an unselected population living in the Basque Country. This approach provides a fast and reliable methodology for determining MnSOD polymorphism in large-scale population studies.

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Poster - 31

Alterations in lipid peroxidation in liver after lead and cadmium intoxication

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Lead and cadmium were usually environmental toxics. Liver is a target organ for both metals (Dudley et al.,1984; Nolan and Shaikh.1992) and they can produce liver toxic effects. One of this, is the induction of the lipid peroxidation; causing an accumulation of free radical (Theocharis et al., 1991; Gurer et al. 1999). We have worked with two groups of Wistar rats: I) treated with distilled water during 30 days (control), II) received lead acetate and cadmium acetate (150 ppm-5 ppm) in drinking water. All animals were 100 days of age at the beginning of treatment, and were sacrificed 30 days later. After that, we extracted the liver and measured some blood parameters (hematocrit, haemoglobin, glucose, ALP, ACP, red and white blood cells count). Furthermore, we evaluated the oxidative damage in liver, in this way lipid peroxidation (TBARS test), catalase activity and total lipid were measured. The results of this study show that lead-cadmium intoxication causes a decrease in the susceptibility of lipid peroxidation in liver, while the catalase activity presents a tendency to increase. We also observed a highly significant decrease in alkaline phosphatase activity. Lead and cadmium can produce an interaction and attenuate the oxidation effect in that cotreatment.

Poster - 32

Pro-oxidant/antioxidant balance impairment in kidney and liver after lead intoxication

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Lead has an oxidative effect in liver and kidney causing an accumulation of reactive oxygen species (ROS) in both organs, that can get to liver-toxicity and renal failure. Also lead produces an increase of hepatic and kidney peroxidation and a descent of certain antioxidant enzymes. In order to study this effect the activity of some physiologic parameters was evaluated and we treated five groups of Wistar rats: I received only distilled water during treatment (control); II-V were treated with lead acetate (300 ppm) in the drinking water; II during gestation period (gestation); III since gestation until weaning (lactation); IV from pregnant period to death (chronic); V only the last 30 days (young). Pups were weighted and were humanely killed at day 100 after birth. After treatments liver and kidneys were removed and kidneys separated into medulla and cortex. The organosomatic index, total proteins, malondialdehyde concentration, total lipids, ALP and catalase activities were measured in the organs. Different blood parameters were also measured: hematocrit, hemoglobin, glucose, red and white cells, ALP and ACP. Lead caused an increase both in liver and renal cortex in lipid peroxidation young group. There was an increase in susceptibility to the lipid peroxidation of chronic and young groups in liver and a decreased in renal cortex fo chronic group, indicating the activation of some type of antioxidant defense. We also observed a decrease in catalase activity in liver of the gestation group, and in all groups in the case of renal cortex. On the other hand, the GSSG/GSH index in gestation, lactation and young groups presented an important increase. Lead caused an alteration of the antioxidant systems at a concentration not expecting to be alarming.

Poster - 33

Oxidative modification of hepatic proteins following exercise: A mechanism of generation of charge variants of HSPs?

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Physical exercise is a relevant factor for the maintenance of health. When performed following appropriate training programmes, it may also promote phenotypic changes leading to improvement of muscle power or aerobic capacity. The cellular stress response is a transcriptional event induced early after exercise that could be involved in its physiological effects. This response is characterized by an increased synthesis and accumulation of a variety of stress proteins (HSPs), some of which may be also modified post-translationally. The present work was directed to analyse whether oxidative stress, derived from an increased production of oxygen (ROS) or nitrogen (RNS) derived free radicals, in addition to be a possible inductor of HSP-synthesis, could also be responsible (at least partially) of the post-translational modifications of HSPs known to take place shortly after exercise. Aimed at this, we have studied the oxidative modifications of proteins in the liver of exercised animals and compared with the result of treating whole liver homogenates with acrolein, a product of lipid peroxidation that generates carbonyl groups when reacting with the amino groups of proteins. The accumulated information indicates that exercise tends to increase the presence of carbonyl groups in liver proteins during the early post-exercise period, with kinetics very similar to that of TBARS formation, increasing them in a statistically significant manner ($p<0.05$) at 48 h post-exercise. The apparent correspondence in the immunohistochemical staining patterns of HSP25 and reactive carbonyl groups in liver sections during the post-exercise period, concentrating around hepatocyte sinusoids and hepatocyte periphery, suggests that both compounds are produced in the same cells and supports the hypothesis of a causal relationship among oxidative stress and HSP25-induction. Modification of a model protein (BSA) with acrolein changed the pattern of electrophoretic mobility and the presence of pI-variants suggesting a second mechanism to add up protein phosphorylation to explain how HSP-variants could be generated.

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Poster - 34

Involvement of iron and NQO1 in the intracellular DAG accumulation- and TAG secretion decrease induced by doxorubicin in rat hepatocytes

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Cytotoxicity of doxorubicin (DOX) has been attributed to intracellular reactive oxygen species (ROS) generated during the drug metabolism. NADPH cytochrome P450 reductase catalyzes the one-electron reduction of DOX, resulting in the formation of semiquinone free radical, which in the presence of O_2 undergoes redox cycling leading to the continuous production of ROS. DOX can also generate free radicals via a non-enzymic mechanism that involves reactions with iron. In contrast, the two-electron reduction of DOX catalyzed by NADPH quinone oxidoreductase (NQO1) renders the less reactive compound hydroquinone. NQO1 has been called a prime defence against quinone cytotoxicity. In previous studies we showed that DOX increased substantially the intracellular levels of diacylglycerol (DAG) and also decreased triacylglycerol (TAG) secretion from rat hepatocytes. The aim of the present work was to determine the involvement of DOX metabolism and free radicals in the DOX-induced response in rat hepatocytes. For this purpose, hepatocytes were exposed to 100 μM DOX in the presence of antioxidants and specific inhibitors of DOX metabolism. The iron chelator deferoxamine inhibited DOX-induced DAG accumulation and was partially effective in preventing the decrease in TAG secretion. The cytochrome P450 inhibitors, metyrapone and proadifen, did not reverted the DOX response, decreasing TAG secretion even more dramatically than DOX alone. Unexpectedly, dicumarol, an inhibitor of NQO1, completely prevented both DAG accumulation and the decrease of TAG secretion. Results suggest that iron and NQO1 play important roles in the observed DOX-induced lipid alterations in rat hepatocytes.

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Poster - 35

N-(4-hydroxyphenyl)retinamide induces apoptosis in leukemia cells through a mitochondrial pathway triggered by ceramide-induced oxidative stress

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Retinoids are recognized as a family of molecules capable of causing impact on many cell functions. The antiproliferative capacity of retinoids and their capacity as selective inducers of differentiation and apoptosis in malignant cells, make them a promising therapeutic agent. *In vivo* trials with animals and *in vitro* assays have shown that the synthetic retinoid, N-(4-hydroxyphenyl)retinamide (4HPR), is a promising chemopreventive and antineoplasic agent for cancer. *In vitro* treatments with 4HPR induce apoptotic cell death in human acute lymphoblastic leukemia cell lines, but not in peripheral blood lymphocytes. We have also established the timing and events that trigger and complete apoptosis. Thus, 4HPR leads to an increase of intracellular ceramide levels, which in turn is caused by sphyngomyelin hydrolysis and *de novo* synthesis. Impairing ceramide accumulation -via specific inhibitors of ceramide synthesis- we have observed that ceramide is the main oxidative stress-inducing factor and the responsible for initiating the apoptotic mitochondrial pathway. Reactive oxygen species (ROS) arise from mitochondrial respiratory-chain: downstream complex I and II, and upstream complex III, just before ubisemiquinone-electron pathway. ROS induce cardiolipin peroxidation, the activation of proapoptotic Bak and Bax proteins and the lost of $\Delta\Psi m$ which involves mitochondrial permeabilization. In consequence, cytochrome *c*, AIF and Ca^{2+} are released to the cytosol, then caspase-3 activated and internucleosomal DNA cleaved. In summary, we have established the molecular events that trigger 4HPR in sensitive human leukemia cells, beginning with the ceramide synthesis and ROS generation at mitochondria.

Poster - 36

Implication of Bcl-2 and GSH in N-(4-hydroxyphenyl)retinamide-induced apoptosis in leukemia cells

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N-(4-hydroxyphenyl)retinamide is a synthetic derivative of retinoic acid which has a great potential as chemopreventive and antineoplasic agent. In previous works we have shown that in sensitive leukemia cell lines, 4HPR-induced oxidative stress triggers the apoptotic stimuli, which in turn activate a caspase-dependent mitochondrial pathway. Herein we have demonstrated that 4HPR exerts diverse proapoptotic efficacy on several tumor cell lines. In such cell lines, the intracellular ROS levels generated by 4HPR are correlated with the sensibility to the retinoid in terms of apoptotic cell death. Basal GSH levels are associated with the ability of tumor cell lines to avoid 4HPR-induced oxidative stress and apoptosis. Moreover, when endogenous GSH are experimentally increased and managed to get normal levels after 4HPR treatments, the cells became more resistant to apoptotic stimuli. When GSH levels are experimentally reduced, the reverse effect is observed. The sensibility/resistance to 4HPR is associated with its ability to cause loss of $\Delta\Psi m$. In this regard we have noted that Bcl-2 overexpression is able to avoid 4HPR-caused $\Delta\Psi m$ disruption, mitochondrial permeabilization and therefore cell death. However, in Bcl-2 overexpressed cells 4HPR generates an oxidative stress. This results indicate that although ROS trigger apoptotic stimuli, $\Delta\Psi m$ disruption plays a central "check point event" in 4HPR-activated mitochondrial pathway. We can conclude that 4HPR proapoptotic effects are related with its ability to generate an oxidative stress. Its activity on the studied cell lines depends on the GSH levels as well as Bcl-2 expression. This one, in turn, regulates ROS-caused $\Delta\Psi m$ disruption. We therefore suggest Bcl-2 and GSH levels as main indicators of tumor cell sensibility/resistance to 4HPR.

Poster - 37

Enhancement of μ-opioid receptor desensitization by nitric oxide in *locus coeruleus* neurons: involvement of reactive oxygen species

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We have previously shown that perfusion with a high concentration of the opioid agonist Met^5-enkephalin (ME) desensitizes μ-opioid receptors in the *locus coeruleus*. Nitric oxide, but not cGMP/protein kinase G pathway, is involved in this process and enhances ME-induced desensitization. The aim of this study was to elucidate, by single-unit extracellular recordings, whether reactive oxygen species contribute to μ-opioid receptor desensitization in the *locus coeruleus* from rat brain slices. First, we studied the enhancing effect of sodium nitroprusside (SNP; 1 mM), a nitric oxide donor, on ME (3 μM)-induced desensitization in the absence or presence of antioxidant agents. Perfusion with the antioxidant agents melatonin (100 μM), trolox (200 μM) or U74389G (10 μM) blocked the effect of SNP on desensitization, whereas they failed to change the desensitization itself (without SNP). Perfusion with the thiol-reducing agent N,N-diethyldithiocarbamide (100 μM) also blocked SNP effect, but it enhanced the desensitization itself**;** a lower concentration of this drug (10 μM) was ineffective. On the other hand, application of the oxidant agent H_2O_2 (1.5 mM) alone was able to enhance ME (3 μM)-induced desensitization and to reduce the effect of a sort application of ME (0.8 μM). Our results suggest that reactive oxygen species may contribute to nitric oxide effect by enhancing agonist-induced desensitization of μ-opioid receptors in *locus coeruleus* neurons.

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Poster - 38

Lipid peroxidation in sporadic Parkinson's disease

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Parkinson's disease (PD) is a neurodegenerative disorder characterized by tremor, rigidity and motor disturbances. The pathogenesis of PD is not completely understood, although oxidative stress was suggested as one of the main factors. The purpose of this study was to compare the endogenous plasma lipid peroxidation as well as the exogenous levels or oxidizability from plasma of sporadic Parkinson's disease (SPD) patients.

Fifty one patients, without relatives with PD, were diagnosed of SPD. All patients were under treatment in the Alteration Movement Unit of the University Hospital of Granada, Spain. The control group consisted of a randomized group of forty subjects, showing acute or chronic osteoarticular pathology, with similar distribution of age and sex. All of them were examined and questioned by an experienced neurologist to rule out signs and symptoms suggestive of early PD, and they had no known relatives with PD. Lipid peroxides were determined in duplicate both at basal state and after co-incubation with hydrogen peroxide and ferrous salt to generate hydroxyl radicals by the Fenton reaction (Agil et al., Clin. Chem. 1995; 41:220).

We found significant differences between control and SPD groups in basal LPO (μmol/l; mean ± SEM)(control group, 2.39 ± 0.12; SPD, 3.19 ± 0.17; $P<0.001$). After incubation with Fe^{+2}/H_2O_2 we also found significant differences (control, 3.90 ± 0.18; SPD, 4.73 ± 0.29; $P< 0.05$).

This study shows that plasma of SPD patients has a lipid peroxidation higher than control group and is also more prone to lipid peroxidation. The oxidative damage of lipids can lead to cell death by a variety of different mechanisms, including the activation of apoptotic phenomena.

Poster - 39

Natural flavonoids morin and mangiferin protect cortical neurons in vitro from excitotoxic insults

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Glutamate excitotoxicity is relevant to acute and chronic neurological impairments. Excitotoxic insults produce oxidative stress and neuronal death. Because of that, we assayed the ability of two flavonoids, mangiferin (1,3,6,7-tetrahydroxy-2-C-β-D-glucoside) and morin (2',3,4',5,7-pentahydroxyflavone), to protect rat cortical neurons from excitotoxicity. Neurons at 8 days in vitro were exposed for 10 minutes to glutamate (1μM–1mM), and cell death was quantified 3 h later using MTT viability assay. Toxicity was half maximal (EC50) at 36 μM of glutamate. Next, neurons were co-treated with flavonoids (1, 10, or 100 nM) during glutamate exposure and left in the medium until the end of experiment. Cellular damage was quantified using calcein AM (1 μM) and free radicals measured at 30, 60 and 180 min after the insult with CM–H_2DCFDA (30 μM). Cell death caused by 50 μM glutamate was decreased by 36.2 ± 2.1% and 34.5 ± 2.5% in the presence of 100 nM morin and mangiferin respectively and, in both cases, free radicals were reduced by 25% after the first hour post-treatment. At higher glutamate concentration (200 μM), cell death was completely abolished by 100 nM morin and significantly reduced by 100 nM mangiferin (10.4 ± 2.4 %) and, in both cases, free radicals were reduced in 10% after 3 hours post-treatment. Taken together, these results demonstrate that morin and mangiferin reduce oxidative stress and are neuroprotective following excitotoxic insults.

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Poster - 40

Ascorbate free radical recycling by the plasma membrane redox chain in synaptic terminals from rat brain

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The plasma membrane redox chain of mammalian cells displays ascorbate free radical (AFR) recycling activity using NADH as electron donor [May, J.M.(1999) FASEB J. **13**:995]. Recently, we have shown the presence of the plasma redox chain in synaptic terminals [Martín-Romero et al.(2002a) J.Neurochem. **82**:604], and its implication in neuronal apoptosis [Martín-Romero et al.(2002b) J.Neurochem. **82**:705; Samhan-Arias et al.(2004) Free Radic.Biol. Med. **37**:48]. In this work we have studied the NADH-dependent AFR reductase activity of plasma membrane vesicles (PMV) prepared from rat brain synaptosomes and its modulation by reduced glutathione (GSH), cytochrome c and coenzyme Q_1. AFR has been measured at 25ºC by EPR in flat quartz cells using a Brucker EMX spectrometer operating at X-band (9.70 GHz) and quantified using TEMPO1 to calibrate the signal intensity. In absence of PMV, 1 mM ascorbate in phosphate buffer (pH 7.2) gives a steady AFR concentration of 0.54±0.04 μM, and in presence of PMV (0.5-1 mg protein/ml) AFR rises to 1.3±0.1 μM. NADH, but not NADPH, promoted a rapid recycling of AFR, in agreement with the fact that NADPH is a poor electron donor for this PMV electron chain [Martín-Romero et al.(2002 a)]. The calculated rate of AFR reduction by PMV was 16±2 nmol/min/mg PMV protein, which is close to the measured rate of the NADH oxidase activity of PMV (20±2 nmol/min/mg PMV protein). GSH (5 mM) increased the rate of AFR reduction, whereas it had no effect on the NADH oxidase activity of PMV, thus, pointing out that GSH promoted additional non-enzymatic recycling of the AFR. Cytochrome c (20 μM) and coenzyme Q_1 (50 μM) stimulated 2 to 3-fold the steady-state level of AFR in presence of PMV, in parallel to the observed 2 to 3-fold stimulation of the ascorbate-dependent NADH oxidase activity of PMV by both cytochrome c and coenzyme Q_1.

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Poster - 41

Dediazoniation of benzenediazonium tetrafuoroborate in an aqueous medium

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It is known that the arenediazonium ions possess mutagenic and carcinogenic capacity and it seems to be well established that the toxicity of the arenediazonium ions is the result of the appearance of very reactive compounds in the dediazoniation process. It has been accepted that the fundamental dediazoniation pathways include the processes that occur through the heterolytic and homolytic mechanisms, where the aryl cation and the aryl radical are the respective intermediate species. As suggested by several authors, both mechanisms may appear simultaneously or competitively, depending on the experimental conditions under which the dediazoniation takes place. We have studied the dediazoniation of the benzenediazonium ion (BZ) in an aqueous medium. The kinetic analyses carried out at different temperatures (20ºC-40ºC) indicate that the dediazoniation process of BZ is a first order reaction in BZ. The analysis of the experimental data gives $A = 2.3\ 10^{15}\ s^{-1}$ and $E_a = 112.5\ kJ.mol^{-1}$ for the Arrhenius equation, and $\Delta H^{\ddagger} = 110.0\ kJ.mol^{-1}$ and $\Delta S^{\ddagger} = 40.7\ J.K^{-1}.mol^{-1}$ for the Eyring equation. The chromatographic results reveal that BZ decomposes through a heterolytic process mediated by the aryl ion. The presence of Cu(I) or Cu(II) salts and ascorbic acid produce a change in the reaction mechanism giving free radicals, which react to form benzene. This result suggests a possible explanation for the localisation of tumours produced by the administration of BZ in rats.

The present work and those entitled "Peroxidative effects exerted by p-hydroxybenzenediazonium ion in two in vitro biological systems" and "A model of Mg-deficient rat used as a reference in the study of the tumour promoting capability of p-hydroxybenzenediazonium ion" were supported by the Regional Government of Andalucia

Poster - 42

Luminol chemiluminescence induced by free radicals derived from *p*-hydroxybenzenediazonium ion

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Chemiluminescence permits not only the evaluation of the end products of reaction of cell constituents with free radicals, but also the observation of the reaction kinetics. Most chemiluminescence methods use only a few chemical components wich are excited by the reaction with free radicals, and have a high quantum yield of photon emission. Luminol is often used as a light source after the excitation by different kinds of free radicals, including peroxyl radicals. Luminol luminescence induced by free radicals coming from azo-compounds under different conditions has been extensively investigated. As an example the azo-compounds-luminol system has been used as a fast and sensitive method to compare an antioxidant potential of new compounds with the commercially used lipid protectors (i.e. butylated hydroxytoluene or Trolox). In the presence of luminol, peroxyl radicals undergo trapping causing the light release. We have analysed the interaction between the free radicals resulting in the dediazoniation of *p*-hydroxybenzenediazonium ion (PDQ) and luminol at pH 7.5. Under these experimental conditions we have already obtained indirect confirmation of the appearance of aryl and peroxyl radicals formed in the PDQ dediazoniation. Although it is well known the limited response obtained from luminol under pH 8, in this study we have detected luminol chemiluminescence induced by the free radicals formed near physiological pH.

Poster - 43

Dietary fat consumption influence angiotensinase activity, lipid profile and hydroperoxides lipid level in plasma of rats.

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Hypertension and hyperlipidemia are frequent causes of cardiovascular disease and major risk factors for atherosclerosis. There is extensive evidence that oxidative modifications of LDL cholesterol play a crucial and causative role in the pathogenesis of atherosclerosis. The fatty acid composition of LDL is influenced by dietary fat and the amount and type of fat in the diet affects the susceptibility of LDL to oxidative damage. Angiotensin II can contribute to atherogenesis through its vasoconstrictor, mitogenic, pro-inflammatory, and pro-fibrotic actions. Growing evidence indicates that Ang II induces its pleiotropic vascular effects through NADPH and NOS-dependent generation of reactive oxygen species (ROS). The function of Ang II is essentially regulated by the action of aminopeptidases (AP) such as GluAP and AspAP. The aim of this study was to evaluate the effects of different diets containing 10 % of sesame oil (S) sunflower oil (SF), fish oil (F), virgin olive oil (O), Iberian lard (L) or coconut oil (C) on the rat plasma levels of total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides, lipid peroxidation and the angiotensinase activities GluAP and AspAP. Enzymatic colorimetric methods were used for the lipids determinations (commercially available kits from SIGMA) and the enzymatic activities were determined fluorometrically using arylamides as substrates. Total cholesterol and LDL levels decreased significantly in rats fed diets containing F and L; HDL increased in the groups O, L and C. The lowest LDL/HDL ratio was obtained in rats fed diet with L. The olive and coconut groups showed the lowest values of peroxidation. Angiotensinase activity decreased with the diet containing fish oil comparing with the lard diet. There were no significant associations between lipid peroxidation values and angiotensinase activity for any of the groups. In conclusion, the present data suggest that the type of fat used in the diet may influence the major risk factors for atherosclerosis by modification of plasma angiotensinase activities, lipid content and oxidative lipid profile.

Poster - 44

Clinical applications of a scoring system for evaluating oxidative stress in humans

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Evaluating oxidative stress (OS) in humans is difficult if we only consider such specific factors as the lipoperoxidation marker or some of the antioxidant enzymes. As a result, we have established a scoring system that contains numerous parameters related to OS. In erythrocytes we analysed haemolysis, glutathione S-transferase, superoxide dismutase, catalase, glutathione peroxidase and reductase. And in erythrocytes and plasma we determined the concentration of thiobarbituric acid reactive substances (TBARS), oxidized glutathione (GSSG), reduced glutathione (GSH) and the GSSG/GSH ratio. We assigned positive or negative values to each parameter depending on whether they increased or decreased with respect to their normal values. And through a mathematical formula we obtained an OS score. This evaluation system was applied to several populations: physiological situations like ageing (A), pathological situations like renal impairment in predialysis (RI) and the evolution of treatment with erythropoietin in RI patients (RI-EPO). We also worked with chronic obstructive pulmonary disease (COPD) patients. In study A, the results showed that in a healthy population older than 65 years of age, the score was 0.04 with no significant differences between other age groups. The score was over 0.5 points higher in men than in women. In the RI population, differences were only significant when we compared the renal impairment population with a control group (1.11 vs. 0.15). In the RI-EPO study, the OS score showed no significant difference between the group after 6 months of treatment (1.12) and the group at the initial time (1.69). The differences were significant, however, between the groups after 6 and 12 months of treatment (1.12 vs. 2.94). In the COPD patients, the OS score was significantly higher than the score in a control population of non-smokers (2.07 vs. 0.14). This scoring system allows us to easily correlate the OS with other clinically related parameters or with illness treatment related parameters.

Poster - 45

Oxidative stress markers in blood and epidermis of samp-8 mice

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The senescence-accelerated mouse (SAMP8) is a suitable model for evaluating the influence of oxidative stress (OS) on the ageing processes. We studied several parameters related to OS on SAMP8 mice and the corresponding control strain SAMR1. The mice were five months old and of both sexes, and both the epidermis and blood were studied.

In comparison with the SAMR-1 mice, in the epidermis of SAMP-8, GST and GR activities increased (50% and 40%, respectively) in both sexes, and GPx and SOD activities increased (18% and 22%, respectively) in females.

In comparison with the SAMR-1 mice, in the erythrocytes of SAMP-8, the GST activity decreased (40% in males and 65% in females), GR activity increased in males (46%), GSH content increased in males (136%), TBARS content increased in females (93%) and GSSG content decreased in females (33%).

We also observed significant differences between the sexes. Thus, in epidermis, male mice of both strains presented higher CAT activity than their corresponding females (48% in SAMP-8 and 112% in SAMR-1); also SAMP-8 males presented higher GSH and GSSG (50%) contents than females. Male SAMP8 mice presented higher erythrocyte GSH content (38%) and higher plasma GSSG content (24%) than females.

These results indicate a higher OS in SAMP-8 mice, with some significant differences depending on gender. The discrepancies observed between erythrocytes and epidermis, especially in relation to GST activity, suggest that there is a greater impact on the erythrocytes of the SAMP-8 strain.

This experimental model appears to be highly suitable for evaluating the efficacy of antioxidant and anti-radical substances.

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Poster - 46

Implication of antioxidant properties of melatonin on its inhibition of cell proliferation in glioma cells

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Increasing evidences implicate oxidative stress in the development and progression of cancer. In fact, reactive oxygen species can act as intracellular messengers that activate mitogenic pathways giving as a result the increase in cellular proliferation. In this work we studied the effect of the antioxidant melatonin, an indolamine produced mainly by the pineal gland, in the proliferation of glioma cells (the most aggressive cerebral tumor). Treatment of cells with melatonin 1 mM produces a decrease in cell number of 33%, 53% and 66% at 24, 48 and 72 hours respectively, accompanied with an accumulation of the cells in the G1 phase of the cell cycle. Antagonists of the known receptors for the hormone did not prevent the effect indicating that it is not receptor mediated. Melatonin 1 mM induces a decrease of intracellular levels of peroxides both at 24 and 48 hours. When we tested another known antioxidant molecules (NAC and trolox) we could check that only the one that decrease intracellular peroxides levels (NAC) was able to induce a reduction in cellular proliferation. Finally, we studied the effect of the different antioxidant in the activity of redox sensitive transcription factors. A reduction in the transcriptional activity of NFκB was observed when cells were treated with both the hormone or with NAC but not with trolox, while no modification in AP-1 transcriptional activity was found. These results suggest that the antiproliferative effect described for melatonin could be mediated by its antioxidant activity.

Poster - 47

Antioxidant vitamins and oxidative stress indexes in pregnant women with intrauterine growth retardation

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Intrauterine growth restriction (IUGR) has a multifactorial ethiology that is not yet completely understood, but recent evidence suggests that oxidative stress is involved in its pathophysiology. The present study was addressed to determine whether IUGR is associated with changes in antioxidant lipophylic vitamins and its potential relationship with an oxidative condition in the mother. Pregnant women with a clinical and ultrasound diagnosis of IUGR were studied at the time of delivery, which occurred at 34.2 ± 3.7 weeks of pregnancy. They were compared to control healthy pregnant women that were studied at the same time of gestation (34.1 ± 2.0 weeks). Blood samples were collected in EDTA. Lipophylic vitamins were measured by HPLC whereas plasma cholesterol and free fatty acid (FFA) as well as other variables related to oxidative stress were determined spectrophotometrically. Plasma fatty acid profiles were determined by gas chromatography after lipid extraction in chloroform-methanol (2:1). Plasma cholesterol and FFA, retinol, γ-tocopherol, α-tocopherol, lycopene and β-carotene were higher in maternal plasma of IUGR than in controls. The differences in retinol, γ-tocopherol and α-tocopherol remained when they were corrected by plasma lipid concentrations. The IUGR group showed higher plasma uric acid levels but unchanged albumin and total protein concentrations. Plasma protein carbonyl, peroxides and MDA levels were higher in IUGR than in control group, indicating an oxidative stress condition. Fatty acid profile of the three groups, expressed as percentage of total fatty acids shows a lower proportion of total n-6 fatty acids but a similar proportion of n-3 fatty acids in IUGR versus controls. Present results show that the IUGR condition is associated with maternal enhanced oxidative stress despite of augmented plasma antioxidant levels, which may result from an effort to compensate for the former condition.

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Poster - 48

Vitamin B_6 nitration and inhibition of vitamin B_6-dependent enzymes by peroxynitrite

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Several aromatic ring-containing coenzymes (such as NAD(P)H and ubiquinone), catecholamines and amino acids (particularly Tyr) belong to the group of small biomolecules showing higher reactivity against peroxynitrite. Due to this, nitrotyrosine formation is a widely used fingerprint of peroxynitrite generation in cells and tissues. In this work we show that the most abundant forms of vitamin B_6 in mammalian cells (pyridoxine, pyridoxamine, pyridoxal and pyridoxal phosphate) display a reactivity against peroxynitrite several-fold higher than that of Tyr. Peroxynitrite has been synthesized as in Gutiérrez-Martín et al. (2002) Free Radic. Biol. Med. **27**:810. The reaction of peroxynitrite with the diverse forms of vitamin B_6 has been monitored by fluorescence quenching and also by the appearance of the nitroaromatic characteristic absorption band centered at 400 nm. As vitamin B_6 in cells is mostly bound to enzymes, the possibility that enzyme-bound vitamin B_6 may be protected against reaction with peroxynitrite deserved to be studied. This possibility has been excluded by using the following enzymes: glutamate-oxaloacetate transaminase (GOT), glutamate-pyruvate transaminase (GPT) and glycogen phosphorylase. Moreover, all these enzymes were found to be strongly inhibited with peroxynitrite (IC_{50} = 5-10 μM), at peroxynitrite concentrations that produce modification of their vitamin B_6 prosthetic form, without significant nitrotyrosine formation (e.g. less than 0.1 mol nitrotyrosine/mol enzyme monomer) and only a low Cys oxidation, which by itself cannot account for the observed inhibition. In conclusion, our results provide a rational basis to reinforce dietary supplementation of vitamin B_6 under pathophysiological conditions that promote peroxynitrite-mediated cell damage, e.g. ischemia/reperfusion syndrome after a heart infarct or inflammation, and suggest the possibility of using nitrated vitamin B_6 as a diagnostic tool to monitor peroxynitrite formation in cells and tissues.

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Poster - 49

A possible role of mitochondria in the prevention by melatonin of cell death induced by oxidative stress

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It has been previously shown that melatonin (MEL) prevents cell death and appearance of oxidative stress indicators in several models of oxidative toxicity, although intracellular pathways remain unknown. It has been suggested that antioxidant properties of MEL may be responsible of this cytoprotective effect. In this work we use as experimental model the prevention by MEL of glutamate-induced cell death in the hippocampal HT-22 cell line. Here, glutamate induces a lineal -although slight- increase of reactive oxygen species (ROS) during the first 8 h of incubation due to glutathione depletion. After glutathione levels drop under a certain threshold (6-8 h after glutamate addition), 12-lipoxygenase (12-LO) is activated inducing an exponential intracellular increase of 1) ROS originated in the mitochondria and 2) calcium intake from the culture medium; and, finally, cell death. We found that MEL prevention of glutamate-induced cell death in HT-22 cells is not accompanied by prevention of glutathione depletion or by any prevention of the initial (first 8 h) increase of ROS. However, the burst of ROS from the mitochondria and the calcium increase caused by 12-LO activation are completely prevented. As MEL fails to prevent 12-LO activation or expression in our experimental model, we consider that mitochondria might likely be the target of melatonin. We also studied the possible implication of MEL receptors on its cytoprotective effect. We found that membrane receptors, ROR/RZR nuclear receptor or Ca^{2+}-Calmodulin complex do not mediate cell death prevention. Finally, we did not find activation of several stress-related transcription factors after glutamate addition or decrease of its constitutive activation when MEL was present in the culture. Given our results, mitochondria appear to be the specific target of MEL in the prevention of glutamate-induced cell death.

Poster - 50

Pindolol is a potent scavenger of reactive nitrogen species. A possible relevant contribution for the reduction of SSRI antidepressant latency

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Pindolol is a β-adrenergic receptor antagonist, which has been shown to enhance and/or accelerate selective serotonin specific reuptake inhibitors (SSRI)-induced antidepressant (AD) action. Although several studies exhibit the pindolol-mediated blockage of pre-synaptic 5-HT_{1A} and 5-HT_{1B} receptors as the responsible for this effect, this matter remains to be completely clarified. On the other hand, it has been demonstrated that inhibition of nitric oxide synthesis in CNS produces anxiolytic and AD-like behavioural effects in a variety of animal paradigms. Importantly, sustained high levels of nitric oxide ($^{\cdot}NO$) may be deleterious to CNS, predominantly due to the formation of peroxynitrite ($ONOO^-$), which is generated via reaction of $^{\cdot}NO$ with superoxide radical ($O_2^{\cdot -}$). Thus, the purpose of the present study was to characterize the putative pindolol scavenging effect on $^{\cdot}NO$, $ONOO^-$, and $O_2^{\cdot -}$, using *in vitro* non cellular systems. The results obtained in the present study clearly show that pindolol is a potent scavenger of $^{\cdot}NO$ and $ONOO^-$. These findings probably contribute for the reduction of SSRI antidepressant latency that have been attributed to pindolol but also may constitute an additional value for this drug when depression is associated with pro-oxidant neurodegenerative diseases.

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Poster - 51

Screening of antioxidant activity of dipyrone and related derivatives using cellular and non cellular *in vitro* assays

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Dipyrone, 4-dimethylaminoantipyrine, isopropylantipyrine and antipyrine are pyrazolinone derivatives, with analgesic, antipyretic and anti-inflammatory activities. It was previously shown that some of these compounds display redox properties. Although these properties may be useful for their therapeutical effects, a thorough and comparative screening of their antioxidant activities against reactive oxidant species remains to be assessed. Thus, the aim of this study was to evaluate and compare the scavenging activity for reactive oxygen species (ROS) and reactive nitrogen species (RNS) using cellular (neutrophil burst) and non-cellular (chemiluminometric and fluorometric assays) methodologies.

The results obtained in this study showed that the tested compounds are scavengers for ROS and RNS, and inhibit the neutrophil burst in a concentration dependent manner, dipyrone and 4-dimethyl-aminoantipyrine being the most potent.

In conclusion, the scavenging activities observed in the present non-cellular *in vitro* assays confirm the putative antioxidant properties for the studied compounds, notably for dipyrone and 4-dimethyl-aminoantipyrine. The results obtained using the non-cellular methodologies were in concordance and reinforced the neutrophil burst inhibitory effects found for these compounds.

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Poster - 52

Antioxidants from sweet pepper: genes, enzymes and non-enzymatic molecules involved in fruit ripening

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In this work, pepper (*Capsicum annuum* L.) fruits from four cultivars (Vergasa, Biela, Galileo and Dulce intaliano) at two different developmental stages were analyzed. In fruits, the activity and gene expression of the following enzymes was investigated: superoxide dismutase (SOD), catalase, gulonolactone-γ-lactone dehydrogenase, ascorbate peroxidase, monodehydroascorbate reductase, glutathione reductase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and NADP-isocitrate dehydrogenase. The ascorbate and polyphenol contents, and the total antioxidant capacity of fruits were also determined.

Results obtained showed that the antioxidant capacity varied in the different cultivars, and this property might be used as a differentiating feature among cultivars. The metabolic parameters more affected by maturation were the SOD isoenzymatic pattern, the ascorbate metabolism and the activity of NADP-dehydrogenases. In all pepper cultivars ripening was characterized by a decrease in the ascorbate content and NADPH generation capacity. Thus, the NADPH-producing enzymes might contribute to the maintenance of an appropriate redox status for the control of the ripening process.

During ripening, fruits did not develop oxidative stress symptoms. However, taking into account the pattern of some antioxidant systems during plant development, in lasting crops there could be imbalances in their oxidative metabolism which could influence the quality and nutritional properties of fruits.

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Poster - 53

Evaluation of etodolac antioxidant activity in a liposomal model system

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It has been well documented that during inflammation, which is basically a defensive phenomenon, reactive oxygen species are produced and uncontrolled release of which leads to tissue damage. Therefore, much attention has been focused in the investigation of antioxidant effect of non-steroidal anti-inflammatory drugs (NSAIDs), besides their anti-inflammatory, analgesic and antipyretic activities.

In this work we have studied the antioxidant activity of the NSAID etodolac, in EPC liposomes. The Fe^{2+}/H_2O_2 system has been used to generate hydroxyl radicals and the water soluble azo-compound AAPH to generate carbon centered radicals by thermal decomposition. The order of effectiveness in avoiding radical chain reactions has been established by using the fluorescent probe DPH-PA. Therefore, peroxidation was monitored by the decay in fluorescence intensity of this probe and by the study of the influence in membrane fluidity. The antioxidant activity of etodolac was also evaluated by the radical DPPH and by the ABTS radical cation decolorization assay. In these methods the NSAID studied act by scavenging the radicals. Besides the investigation of the etodolac ability to inhibit lipid peroxidation or to protect the damage by free radicals, another aim of this work was to study if this drug is able to inhibit damage in biomolecules such as DNA. Once progressive accumulation of oxidative damage to DNA is though to be central to the development of a number of diseases and may play a critical role in the ageing process. For this purpose, we used 2'-deoxyguanosine and investigated if the NSAID inhibited the oxidation of this DNA base to 8-hydroxi-2'-deoxyguanosine induced by Fe^{3+}-EDTA/H_2O_2/ascorbic acid.

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Poster - 54

NSAIDs' antioxidant activity: any influence in therapeutic and toxic effects?

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Non-steroidal anti-inflammatory drugs (NSAIDs) are the principal drug treatment for rheumathoid arthritis, and their anti-inflammatory and gastrointestinal bleeding effects may be partly due to their ability to interfere with free radical-mediated reactions in addiction to the extensively studied inhibition of cyclooxigenase (COX). Indeed, reactive oxygen species (ROS) are produced during inflammatory response as a consequence of COX activity and it has also been established that oxidative stress is an important component of gastrointestinal ulceration. To gain a deeper insight into the anti-radical properties of these drugs, we have studied their antioxidant activity in a membrane model which, when subjected to oxidative stress, is able to generate typical lipid peroxidation cascade (oxy and lipid radicals) that takes place in tissues during the inflammatory reactions. Thus, the study was undertaken in EPC liposomes where the peroxidative degradation of a probe (DPH-PA) was initiated by two different types of radicals (hydroxyl and peroxyl radicals). Peroxidation was simultaneously indicated by a decrease in the probe's fluorescence and by anisotropy measurements which detect subtle changes in membrane rigidity. Antioxidant activity was also assessed by the general screening DPPH and ABTS free radical methods which evaluate the drugs' radical scavenging properties

Apart from membranes, DNA is also a cellular component which is particularly susceptible to oxidative damage and may be involved in cancer and neurodegenerative diseases in which NSAIDs have been reported to exert beneficial effects. Therefore, the inhibition of 2'-deoxyguanosine oxidation to 8´-hydroxy-2'-deoxyguanosine was also accessed by HPLC.

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Poster - 55

Effects of alkylamides of cinnamic and hydrocinnamic acids on lipid peroxidation of liposomes

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There is increasing evidence that free radical-induced oxidative damage may play a role in the pathogenesis of many neurological, particularly neurodegenerative, diseases. The brain is especially sensitive to oxidative damage because of its high content of readily oxidized fatty acids, high use of oxygen and low levels of antioxidants. It is believed that exogenous antioxidants could be very effective in diminishing the cumulative effects of oxidative damage. The therapeutic use of most of the antioxidants investigated is limited since they do not cross the blood brain barrier (BBB). Therefore, any novel antioxidant compounds designed for potential neuroprotective treatment in neurological disorders should have a high degree of lipophilicity in order to penetrate the BBB.

In this context we have designed new antioxidant molecules based on compounds with well know antioxidant properties, but with increased lipophilicity conferred by an additional alkyl chain. In order to reach this goal, hexylamides of cinnamic and hydrocinnamic acids have been prepared and their effects on liposomes lipid peroxidation, induced by AAPH, evaluated.

From the results obtained it is possible to infer that the synthesized amides maintain or even increase the antioxidant activity relatively to the precursor acids. Furthermore, the hexylamides of caffeic and hydrocaffeic acids showed to be the most active of all the amides investigated. The results obtained could contribute to the rational design of neuroprotective antioxidants effective in the treatment of neurological disorders.

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Poster - 56

Expression of antioxidative enzymes in response to environmental stresses in pea plants

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In plants, the ascorbate-glutathione cycle is an important antioxidative system regulating the cell level of H_2O_2 in conjunction with catalase [1]. This cycle is present in different cell compartments (chloroplasts, cytosol, peroxisomes and mitochondria) and is formed by the enzymes glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and ascorbate peroxidase (APX) [1,2].

Using leaves from pea plants subjected to different types of environmental stress (high light intensity, high and low temperature, mechanical wounding, Cd and the xenobiotic 2,4-D) the expression of GR, MDHAR, catalase, and the NADPH-producing enzyme isocitrate dehydrogenase (ICDH), was studied by semi-quantitative RT-PCR.

Results obtained showed a different expression pattern for each of the enzymes studied. The maximum induction of cytosolic and chloroplastic GR was produced by cold stress, whereas high light intensity brought about the maximum inhibition. In contrast, MDHAR showed a highest expression by cold, mechanical wounding and 2,4-D stress. Cold stress and mechanical wounding induced the cytosolic NADP-ICDH but its expression was not inhibited by any of the other stress conditions assayed.

[1] Noctor G and Foyer CH (1998) *Annu Rev Plant Physiol* **49**, 249-279
[2] del Río LA et al (2002) *J Exp Bot* **53**, 1255-1272

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Poster - 57

Phospholipid hydroperoxide glutathione peroxidase (PH-GPx) expression is downregulated in poorly differentiated breast invasive ductal carcinoma compared to the adjacent non-tumoral breast ductal tissue

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Phospholipid hydroperoxide glutathione peroxidase (PH-GPx or GPx4) is an essential antioxidant protein with potential implications in cell proliferation and apoptosis. Previous data also suggest a role for oxidative stress in breast carcinogenesis. In this context, we evaluated the relative expression of PH-GPx in samples of human breast invasive ductal carcinoma and samples of their non-tumoral adjacent breast tissue from 34 patients. We quantitated PH-GPx mRNA by quantitative PCR and analyzed the results using the standard Comparative C_T method (Applied Biosystems®, Foster City, CA). The results show that PH-GPx expression is down-regulated in the tumoral samples when compared with the non-tumoral adjacent breast tissue samples. Interestingly, a positive correlation was found between the most down-regulated cases and the less differentiated tumors (P= 0.0043). Our results suggest that PH-GPx expression is needed to control the redox status in breast tissues. Additional studies are needed to evaluate the functional implications of PH-GPx in breast carcinogenesis.

Poster – 58

S-Nitrosylation of Hsp90 promotes the inhibition of its ATPase and eNOS regulatory activities

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Nitric oxide is implicated in a variety of signaling pathways in different systems, notably in endothelial cells. Some of its effects can be exerted through covalent modifications of proteins and, among these, increasing attention is being paid to S-nitrosylation as a signaling mechanism. Using a specific proteomic approach, we were able to identify a number of proteins that are S-nitrosylated in endothelial cells. Among them, we identified the molecular chaperone Hsp90, which is also one of the proteins involved in the activation of endothelial nitric oxide synthase (eNOS).

In this work we show by a variety of methods that Hsp90 is a target of S-nitrosylation, and identify the susceptible cysteine residue in the region of the C terminal domain that interacts with eNOS. We also show that the modification occurs in endothelial cells when they are treated with S-nitroso-L-cysteine, and when they are exposed to eNOS activators. Hsp90 ATPase activity and its positive effect on eNOS activity are both inhibited by S-nitrosylation. Together, these data suggest that S-nitrosylation may functionally regulate the general activities of Hsp90, and provide a feedback mechanism for limiting eNOS activation.

Poster - 59

Moderate exercise up-regulates the expression of antioxidant genes and of transcription factors for mitochondrial biogenesis. Oral antioxidant administration prevents it

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Exhaustive exercise generates excessive amounts of oxidative free radicals that overwhelm cellular antioxidant defences and may cause tissue damage. However there is considerable interest in the potential of these mediators to regulate muscle adaptation to exercise. This is one of the oldest postulates in the field, dating back to the suggestion that free radicals produced in exercising muscle might stimulate mitochondrial biogenesis and the expression of genes for antioxidant enzymes. The aim of this study is to elucidate the role of the free radicals generated in moderate physical exercise, in the expression of antioxidant genes and of transcription factors for mitochondrial biogenesis. Twenty male Wistar rats were randomly divided into four groups: sedentary controls (n=5), exercised (n=5), exercised treated with C (n=5) and exercised treated with allopurinol (n=5). Allopurinol acts as an antioxidant because it inhibits xanthine oxidase, an important generator of free radicals in exercise. Where indicated animals were subjected to moderate exercise training five days a week during three weeks. Our results show that moderate exercise up-regulates the expression of antioxidant enzymes associated with longevity, such as Mn-SOD and GPx. We also found that moderate exercise up-regulated the expression of NRF-1 that is a key transcriptional activator of nuclear genes encoding mitochondrial enzymes and Tfam, which stimulates mitochondrial DNA transcription and replication. However, supplementation with vitamin C or allopurinol during training prevented all of these adaptations. We conclude that the usual practice of recommending antioxidant supplements before exercise should be seriously questioned. Oral antioxidant supplementation is very likely to be useful before competition when exercise is likely to be exhaustive, and damaging, but not when training.

Poster - 60

Xanthine oxidase: A significant source of oxidative stress in aging

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Aging is a natural process that is characterized by a gradual and general decline in physiological functions. The role of reactive oxygen species and its effects on aging was confirmed by D. Harman which proposed in 1954 the "free radical theory of aging". In 1980, Miquel proposed mitochondria as the principal organ which induces aging in cells. Today, the aging process is considered a chronic oxidative stress process.

Xanthine Oxidase (XO) is one of the major sources of reactive oxygen and nitrogen species (ROS, RNS). Its activity increases in different pathophysiological processes like ischemia-reperfusion.

We studied the XO activity and its genomic expression in plasma, aorta, muscle and liver of young (3 months) and old (24 months) Wistar rats.

In plasma from young rats XO activity is 34.65 % higher in male animals. In plasma from old animals XO is also 36.94 % higher in male rats. XO activity in plasma old female rats was found a 43.91 % higher than in their young counterparts. In a similar fashion plasma XO activity from male old rats was 46.36 % higher than in young animals.

XO activity in tissues of old rats is 107 % higher in aorta, 78 % in muscle and 166 % in mammary gland. The genomic expression of XO increases in mammary gland from old rats 84 % and 12.5 % in liver.

Xanthine Oxidase one of the principal source of free radicals could be marker in aging and its inhibition could influence survival rate in different species.

Poster - 61

Free radical scavenging capacity of Manto Negro grape stalks

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The antioxidant activity of wine is related with a potential preventing effect of coronary heart disease and there is medical evidence that the moderate consumption of wine during meals, typical of the Mediterranean diet, is beneficial to health. The antioxidant activity of wines has been related to their polyphenolic constituents and is mainly based on their free radical scavenging capacity. Part of the polyphenolic compounds present in the grape pass to the wine in the winemaking process but, independently of this transfer, a high proportion remains in the vinification residues or wastes.

Manto Negro is the major red grape variety native from Mallorca used in local wine industry. Stalks are considered by-products without any economic value and in this study they are treated as a possible source of natural antioxidants.

Manto Negro stalks total polyphenols were extracted and quantified by the Folin-Ciocalteau method. Total catechins and procyanidines were also determined. The extracts were used to measure the antioxidant activity. The free radical scavenging activity was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH·) method. Vitamin E was used as standard and compared with the stalks values in terms of the antioxidant activities in the DPPH· method. The results demonstrated that stalks have high levels of total polyphenols and about the 70 % of these are catechins and procyanidines. On the other hand, Manto Negro stalks present a very high free radical scavenging activity, which is in agreement with the results mentioned above.

The values obtained encourage us to pursue in investigating this material as a source of natural antioxidants with possible beneficial effects to health.

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Poster - 62

Study of the structure-activity relation of flavonoids as lipoxygenase inhibitors

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The pharmacological effect of flavonoids is mediated by their antioxidant properties as prooxidant enzymes inhibitors or as radical scavengers. The antioxidant properties of flavonoids result from the formation of stable radicals due to the electronic delocalisation in the rings A, B and C. Also, it is known that the biological properties of flavonoids are related with the presence/absence of C2-C3 double bond, with the position of hydroxyl groups or with the planarity of the molecular structure. Although, until now, it is accepted the health beneficial effects of flavonoids, it has been reported a lot of results related to prooxidant and mutagenic properties in contrast with others in which the antioxidant and antimutagenic properties of flavonoids are clearly demonstrated. Recently, several studies based on theoretical investigations using semiempirical methods have contributed efficiently to the knowledge of the relation between the structural and electronic properties of flavonoids and the antioxidant effects shown by these molecules. From this point of view, it is of great interest the study of the interaction of flavonoids with prooxidant enzymes, as for instance, lipoxygenase, an enzyme that has been considered a target of the health preserving effect of flavonoids. In this work we have studied the correlation between the molecular properties of four flavonoids used as model, quercetin, catechin, taxifolin and luteloin, and the ability shown by these molecules as lipoxygenase inhibitors. When the data obtained from the evaluation of the potential energy surface of flavonoids, as a function of the torsional angle between rings C and B, were compared with the lipoxygenase inhibitory efficiency, we obtained a clear correlation between the planar character of the flavonoid molecule and their inhibitory capacity. It is known that frontier orbitals are related with the scavenging free radical properties of molecules. We have checked the role of frontier orbitals on the efficiency of flavonoids as lipoxygenase inhibitors. Our results clearly show that the delocalization of lowest unoccupied molecular orbitals (LUMO) is correlated with the degree inhibitory efficiency exerted by assayed flavonoids.

Poster - 63

Physiological concentrations of 17β-estradiol and genistein exert their antioxidant action by induction of antioxidant gene expression in a mammary gland tumour cell line

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Females live longer than males. The higher levels of oestrogens in females protect them against ageing by up-regulating the expression of antioxidant longevity-related genes. Oestradiol and genistein share chemical properties which confer antioxidant features to these compounds. The low concentration of them makes it unlikely that they exhibit significant antioxidant capacity in the organism. The aim of this study is to elucidate the mechanism through which oestrogens and phytoestrogens up-regulate the expression of antioxidant enzymes. We have used a mammary gland tumour cell line (MCF-7). MAPK was determined by western blotting, translocation of p50 subunit of NF-κB to the nucleus in nuclear extracts by ELISA, expression of antioxidant enzymes by quantitative real-time RT-PCR, and peroxide levels was measured fluorimetrically. Our results show that physiological concentrations of oestrogens and concentrations of genistein equivalent to those present in blood of oriental people, activate the MAPK pathway. These, in turn, increase p50 subunit of NF-κB in nuclear extracts from cells treated with oestrogens or phytoestrogens. The Mn-SOD and the GPx promoter region contain putative NF-κB-binding motifs. Thus, activation of NF-κB by oestrogens subsequently activates the expression of Mn-SOD (3.5 fold) and GPx (2.2 fold), but genistein is only capable to activate Mn-SOD expression (2.9 fold). This antioxidant protection is reflected in the lower peroxide levels and is prevented when cells are co-treated with the MAPK phosphorylation inhibitor UO126. We conclude that oestrogens and phytoestrogens up-regulate expression of antioxidant enzymes via the MAPK activation, which in turn activate the NF-κB signalling pathway.

Poster - 64

Nuclear glutathione regulates the telomerase activity in 3T3 fibroblasts

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Telomerase is constituted by RNA and different proteins that participate in the synthesis of telomeric DNA *de novo*, preventing the shortening of telomeres during DNA replication. It has been demonstrated that an alteration in the regulation of telomerase activity is implicated in cancer and aging. Reduced glutathione (GSH) is the most abundant non enzymatic antioxidant in the cell that has a central place in the control of vital cell processes of great diversity including cell proliferation. GSH is crucial for DNA synthesis, regulation of nuclear matrix organization, nuclear signal transduction and gene transcription. Furthermore, there is growing evidence that shows the importance of GSH compartimentation and the existence of ATP-dependent nuclear GSH transport.

The objective of our study was to investigate a possible relation between the nuclear GSH and the regulation of telomerase activity. We have measured the level of total GSH (GSHt) by spectrophotometry, the cellular distribution of GSH by confocal microscopy, telomerase activity (TA) by TRAP and cell cycle by flow citometry. At 24h in culture, the level of GSHt is 18.3±2.4 nmols/10^6 cells, comparing to 4.7±1.8 at 6 h, and TA at 24 h has 170 % of the value measured at 6 h. This peak of GSHt and TA precedes the phase of the exponential cell growth. At 48-72 h, 40 % of the cells is in S+M/G2 phase of the cell cycle and levels of GSHt and TA are progressively lower. Furthermore, our studies of the GSH distribution throughout the cell cycle show a growing tendency of GSH concentration in the nucleus during the first 24 h of culture, maintained until 48 h, which coincides with exponential phase of cell growth. At 5 days, as cells reach confluence and 80 % of cells is in Go/G1 phase, the distribution of GSH is completely homogeneous.

We conclude that GSHn regulates the telomerase activity in 3T3 fibroblasts and determines the rhythm of the growth of cell culture.

Poster - 65

Screening plant extracts for determining their radical scavenging activity

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An increasing number of free radicals or decreasing radical defences (antioxidant/scavenger activity and enzymes) are implicated in etiopatology and complications in many diseases and aging. Targeting free radicals through new compound systems, without side effects (or limited ones), capable of being used in human nutrition and food systems, has proven to be useful[1]. The use of integral extracts of plants (IEP) is an approach for search compounds with significant antiradical activity. The radical scavenging activity, of ethanol and ethanol/acetone extracts of some plants used in Portuguese popular medicine and food, was evaluated in a system with azobisisobutyronitrile (2 mmol) and galvinoxyl (0.2 mmol). The rate of galvinoxyl decomposition was followed by measuring the decrease in its absorption at 420nm. The free radicals will be blocked by the IEP (1 ml) and their antiradical efficiency was defined as a Protection Index (PI). All ethanol extracts have a firm radical scavenging activity, which lasts over 125 hours. The ethanol extracts from *Silybum marianum* L. and *Rosmarinus officinalis* L. are the most efficient ones. When different stages of the harvest and parts of the used plant are compared, several IEP presents prooxidant activity according to the PI. Total phenolic contents of IEP were measured and expressed as caffeic acid equivalence (CAE). The highest CAE value detected was 4.8 mg (32.1 %) per ml for *Hypericum perforatum* L. (ethanol extract). No direct correlation was observed between total phenolic contents and radical scavenging capacity or prooxidant effects**.**

(1) Zheng, W. and Wang, S. Y. "Antioxidant Activity and Phenolic Compounds in Selected Herbs". *J. Agric. Food Chem.* 2001, 49, 5165-5170.

Poster - 66

Role of TNF-α, xanthine oxidase and MAP kinase in glutathione depletion in experimental acute pancreatitis

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Pancreatic injury triggers two major pathways involved in the systemic effects of severe acute pancreatitis: pro-inflammatory cytokines and oxidative stress. Aims: To assess the effects of inhibiting both TNF-α production and xanthine oxidase activity on the inflammatory response and to assess the role of MAP kinases and TNF-α receptors in glutathione depletion (GSH) in acute pancreatitis (AP). Methods: Two models of pancreatitis were performed. In rats, AP was induced by intraductal infusion of 3.5 % sodium taurocholate. In TNF-α receptor 1 and receptor 2 knockout mice, AP was induced by seven injections of cerulein (50 μg/kg), - an analog of CCK-. We examined whether treatment with oxypurinol - a specific inhibitor of xanthine oxidase- and/or pentoxifylline - an inhibitor of TNF-α production- affects GSH depletion or oxidation in pancreas and pancreatic MAP kinase phosphorylation in rats. In knockout mice, GSH levels were measured in pancreas. Results: In rats, early GSH depletion occurred in pancreas but not oxidation. Oxypurinol prevented later GSH oxidation and p38 phosphorylation in pancreas. Pentoxifylline prevented ERK 1/2 and JNK phosphorylation and prevented GSH depletion in pancreas. Combined treatment with oxypurinol and pentoxifylline almost completely abolished MAP kinase phosphorylation and GSH depletion or oxidation in pancreas. Cerulein-induced AP showed GSH depletion in pancreas with no differences between TNF-α receptor 1 or 2 knockout mice and wild type. Conclusions: Simultaneous inhibition of TNF-α. production and xanthine oxidase activity greatly reduced glutathione depletion and oxidation in acute pancreatitis, this effect was associated with blockade of the three major MAP kinases. TNF-α receptors seem not to be involved in GSH depletion in experimental acute pancreatitis.

Relationship between telomerase activity and cell redox status

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Telomerase activity regulation is essential to understand its function in the maintenance of the telomere length. Furthermore, there is a relationship between telomerase activity and aging (1). In a similar fashion, telomerase knockout mice are more resistant to xenobiotic induced carcinogenesis (2). Glutathione is the main non-enzymatic antioxidant inside the cells (3) and is implicated in the defence against the deleterious effects related to aging, mainly at mitochondrial level.

The main purpose of this work was to determine the relationship between telomerase activity and cell glutathione levels. We performed experiments *in vitro* using a cell line (3T3 fibroblasts) and *in vivo* using telomerase knockout mice.

It has been shown a correlation between telomerase activity and cell glutathione levels in cultured cells (4). Here we demonstrate that telomerase activity is regulated by the redox status of the cell. When cells are depleted of glutathione, telomerase activity is diminished. Replenishing cells with glutathione monoethylester reverts this decrease. Moreover, in cell protein extracts, telomerase activity is regulated by changes in the glutathione redox status around GSH:GSSG ratios similar to the physiological conditions (from 10:1 to 100:1). Moreover, telomerase knockout mice have increased levels of glutathione independently of the telomere length. This is due to the fact that telomerase knockout mice over express γ-glutamyl cystein sinthetase, the key enzyme for the synthesis of glutathione.

We conclude that telomerase activity is strongly related to the glutathione levels in cells.

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Poster - 68

Blood antioxidant markers in Azorean subjects with coronary heart disease

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Atherosclerosis and related vascular diseases are the first cause of morbidity and mortality in Portugal, including the Azores' Archipelago. Epidemiological and experimental studies infer that a disturbed pro-oxidant/antioxidant balance can play a central role in atherosclerosis. In this study blood activities of antioxidant enzymes-superoxide dismutase (SOD) and glutathione peroxidase (GPx)- as well as plasma total thiols, serum vitamin E and ceruloplasmin were evaluated in subjects from Ponta Delgada with coronary heart disease submitted to percutaneous revascularization (PCI). Significant decreases in both whole blood GPx activity (14 %) and serum vitamin E concentration (10 %) were found in these patients as compared to the control group constituted by apparently healthy subjects. When analysed by gender only the male groups (patients and controls) exhibited significant differences in serum vitamin E levels. No sex-related differences were observed in GPx activity. Results suggest a depletion in selective antioxidant defense mechanisms in PCI patients, since erythrocyte SOD activity and plasma total thiols were unchanged.

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Poster - 69

Serum paraoxonase activity related to Q192R polymorphism and circulating HDL in post-menopausal women

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Human paraoxonase 1 (PON1), encoded by *PON1*on chromosome 7q21.3, is an arylesterase serum enzyme associated with high-density lipoproteins (HDL). PON1 protects low-density lipoprotein (LDL) from oxidation, probably as a result of its ability to hydrolyze specific oxidized lipids. The common coding polymorphism Q192R has been described to influence PON1 activity. The aim of this work is to determine in post-menopausal women the relationship between PON1 activity and: (1) serum HDL-cholesterol (HDLc) and apo A-I levels, and (2) different Q192R genotypes. HDLc was quantified by commercially available enzymatic kits and apo A-I by immunonephelometry. PON1 activity was measured spectrophotometrically, using paraoxon (paraoxonase activity) and phenylacetate (arylesterase activity) as substrates. Polymorphisms were detected by the polymerase chain reaction-restriction fragment length polymorphism assay. Results showed that PON1 arylesterase activity correlated significantly with serum apo A-I levels. However, PON1 paraoxonase did not correlate with HDLc or apo A-I. Paraoxonase activity was genotype-associated according to the order RR>QR>QQ. R allele carriers showed lower arylesterase activity in the studied population.

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Poster - 70

Development of new phenolic derivatives as promising antioxidant/anticancer agents

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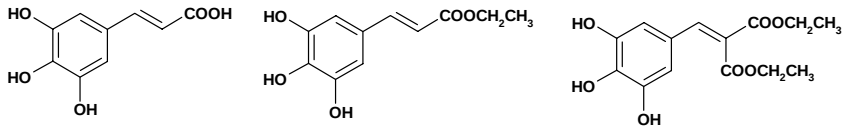
Over the past few years, several phenolic acid derivatives have been synthesised in our group, in view of studying their antioxidant profile. Apart from the antioxidant activity displayed by these compounds, due either to a radical scavenging mechanism or to an inhibition of the enzymatic systems responsible for free radical generation, they were also found to possess anticancer properties. Once oxidative damage appears to be closely related to carcinogenesis, it seems essential to correlate the antioxidant/anticancer potential of a series of phenolic acid analogues. Moreover, the understanding of the *structure-activity relationships* ruling the biological functions of this kind of compounds is of the utmost importance for the development of new chemopreventive/therapeutic agents.



The design and synthesis of pyrogallol type derivatives, their conformational analysis by theoretical (*ab initio*) methods, and the results of the *in vitro* evaluation of their antioxidant/anticancer activities will be presented.

C. Siquet thanks FCT and FSE for the fellowship (SFRH/BD/7005/2001)

Poster - 71

Synthesis of lipophilic phenolic derivatives and evaluation of their antioxidant profile against lipid peroxidation

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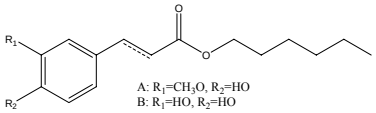
With increasing evidence suggesting the involvement of oxidative stress in various disorders and diseases, the role of antioxidants *in vivo* has received much attention. In this project, novel and potentially therapeutic antioxidants, that combine the radical-scavenging ability of a caffeic or ferulic acids-like head group with a lipophilic chain similar to that of vitamin E, have been prepared.

This study will also allow the understanding of the structural basis of their chain-breaking properties, aiming specifically at elucidating the effects of substituents in the antioxidant properties of phenolic compounds. For this, a series of derivatives were synthesised and examined for their capacity to scavenge radicals (ABTS, DPPH) and inhibit lipid peroxidation in liposomes subjected to the peroxidizing action of AAPH.



From the data obtained it was concluded that the new antioxidants exerted higher reactivity toward radicals and lipid peroxidation than their hydrophilic counterparts. Reference antioxidants like octyl gallate, vitamin E, the main lipid-soluble antioxidant in biological membranes, and trolox were used in the study.

Orientation of the head group as well as total lipophilicity is being evaluated as important determinants of antioxidant efficacy.

C. Siquet thanks FCT and FSE for the fellowship (SFRH/BD/7005/2001).

Poster - 72

Does resveratrol have a protective role against proatherogenic activity of peroxynitrite?

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Atherosclerosis is a chronic inflammatory condition associated to oxidative stress. In fact, the hypothesis of oxidative stress has gained considerable support, not only by the crucial role of oxidized LDL in the atherogenic process, but also by the stimulation of reactive oxygen and nitrogen species release from endothelial, vascular smooth muscle and immune cells. The simultaneous generation of nitric oxide and superoxide favours the production of peroxynitrite, a very toxic reaction product. In this context, peroxynitrite has been implicated in atherogenesis by its ability to oxidize LDL and to induce cell death. Resveratrol is a natural phytoalexin believed to be responsible for the red wine cardiovascular benefits due to a set of pharmacological properties, including antioxidant activity. Thus, the aim of this study is to explore the resveratrol ability to inhibit peroxynitrite-mediated LDL oxidation and the underlying antioxidant mechanism, as well as the potential deleterious effects of peroxynitrite on cellular viability of BAEC and the putative protection afforded by resveratrol. This compound interacts with peroxynitrite by an oxidative mechanism, inhibiting LDL apoprotein modifications as detected by a decrease in peroxynitrite-mediated apoB charge alterations and carbonyl groups formation. Moreover, such protection is not altered by physiological concentrations of bicarbonate. On the other hand, peroxynitrite (400 – 600 μM) is cytotoxic to BAEC and the resulting increase in DNA fragmentation as well as the nuclei condensation and fragmentation suggest that a programmed cell death pathway is triggered. The potential protective role of resveratrol on peroxynitrite-mediated injury in endothelial cells is under study.

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Poster - 73

Hyperthermia-induced toxicity in freshly isolated mice hepatocytes. Involvement of oxidative stress and cellular signalling through the activation of heat shock factor 1 (HSF1)

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The hepatotoxic effects of hyperthermia have been associated with the over-generation of reactive oxygen species (ROS) and depletion of cellular antioxidants leading to oxidative stress. There are multiple mechanisms or processes for cell and tissue protection against ROS. In addition to direct antioxidant defence systems, the genes for heat shock proteins (HSPs) can also be upregulated in response to cellular trauma, resulting in cellular protection and enhanced survival. This effect is mediated by the activation and DNA binding of heat shock factors (HSF), notably HSF1.

The aim of the present work was the implementation of a suitable and simple cellular model for the study of hyperthermia-induced hepatotoxicity and evaluation of cellular signalling through the induction of HSF1. Freshly isolated mouse hepatocytes obtained from Charles River CD1 male adult mice were incubated under normothermia (37 ºC) or mild hyperthermia (41ºC) for 4 hours. Under the present experimental conditions, hyperthermia caused oxidative stress in a time-dependent manner. It was observed a significant induction of lipid peroxidation, depletion of reduced glutathione, formation of oxidized glutathione, and loss of cell viability (≈ 70% mortality at 4 hours of incubation). Additionally, it was also found that hyperthermia was capable of inducing a heat shock response, reflected by the presence of HSF1, already observed at the second hour of incubation, with a maximum effect obtained at 4 hours.

In conclusion, in the present study it was demonstrated that the present cellular model is suitable for the evaluation of hyperthermia induced oxidative stress and transcriptional activation, specifically of HSF1.

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Poster - 74

3,4-Dihydroxyphenylacetic acid modulates the toxicity induced by nitric oxide in PC-12 cells. The critical role of GSH

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The neurotoxic effects of nitric oxide ($^{\bullet}$NO) are well established but the precise mechanisms underlying toxicity have not been clearly understood yet. We have previously shown that, in isolated mitochondria, $^{\bullet}$NO and 3,4-dihydroxyphenylacetic acid (DOPAC) a metabolite of dopamine, induced a synergistic inhibition of mitochondrial respiratory chain that ultimately could lead to mitochondrial dysfunction, an event that may be a central theme in neurodegeneration. In the present study, we devise to establish whether the $^{\bullet}$NO/DOPAC interaction could induce toxicity in PC-12 cells, often used as a neuronal cell model.

Exposure of cells to $^{\bullet}$NO donor SNAP induced cell death in a time- and concentration-dependent manner. DOPAC potentiated the toxic effect promoted by $^{\bullet}$NO. Loss of cell viability was accompanied by depletion of intracellular glutathione (GSH). Modulation of intracellular thiol status with GSH ethylester, DTT and NAC prevented the loss of cell viability induced by $^{\bullet}$NO/DOPAC. Also, co-incubation of cells with catalase render cells resistant to death, suggesting the involvement of H_2O_2 in the toxic pathways trigger by $^{\bullet}$NO and DOPAC.

Collectively, these findings suggest that NO and DOPAC impose an oxidative/nitrosative stress leading to cell death that may be overcome by up-regulation of intracellular GSH pool. These results may have significance for the prevention of neurodegeneration associated with Parkinson´s disease where GSH depletion and $^{\bullet}$NO overproduction have been documented.

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Poster - 75

Antioxidant properties of a *Hypericum perforatum* ethanolic extract and its isolated phenolics

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Oxidative stress has been implicated in the development of several chronic diseases. Recently, we showed *Hypericum perforatum* (HP) extracts to be effective radical scavengers and to possess antioxidant proprieties. To further characterize the antioxidant proprieties HP extracts we used a total ethanolic extract (TE) and pure compounds isolated from the TE.

TE was able to scavenge NO released from sodium nitroprusside (78.69±1.30 %, with 10 μg dwb.ml^{-1}) and to scavenge HOCl in a dose-dependent manner (10.22±2.08 %, with 10 μg dwb.ml^{-1}). TE was effective in scavenging DPPH (EC_{50}=49.30±1.05 μg dwb.ml^{-1}). The most effective compounds present in TE were quercetin-type flavonols, hyperoside being the most effective (6.38±1.06 μM) and quercitrin the less effective (12.96±1.10 μM). Chlorogenic acid had an EC_{50} of 20.29±1.04 μM. The flavones biapigenin and amentoflavone, and hypericin were poor scavengers of DPPH (inferior to 30 %, at a maximum concentration tested). Lipid peroxidation, induced either by ascorbate/Fe^{2+} or by AAPH peroxyl radical, was significantly inhibited in the presence of the TE. TE exhibited an EC_{50} value of 27.67±1.26 μg dwb.ml^{-1} for ascorbate/Fe^{2+}, almost half of that observed for AAPH induced lipid peroxidation. Compounds that efficiently protected against lipid peroxidation were also more effective in ascorbate/Fe^{2+} than in AAPH induced lipid peroxidation. Quercetin and kaempferol were the most effective compounds in reducing ascorbate/Fe^{2+} induced lipid peroxidation (EC_{50}=0.12±0.04 and 0.69±0.1 μM, respectively), whereas hyperoside was the most effective against AAPH induced lipid peroxidation (EC_{50} = 11.47±1.76 μM). Hyperoside was also the most effective against the radical DPPH, which seems to correlate both activities. In conclusion, TE exhibited strong antioxidant activity, protecting at several levels in different models of oxidative stress. From the several compounds tested, quercetin and hyperoside were the most active.

Poster - 76

Xanthine oxidase derived free radicals are responsible for NF-κB activation through MAP kinase pathway in diabetes

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Oxidative stress has been involved in the pathogenesis of diabetes. Hunt et al (1988) suggested that auto-oxidative glycosilation is important in explaining free radical formation and protein damage in diabetes. Jain et al (1999) have proposed that ketone bodies, especially acetoacetate, are involved in free radical formation in type I diabetes. We propose an enzymatic mechanism to explain the increased generation of free radicals in diabetes. In 2002 we confirmed that there is oxidative stress (oxidation of glutathione and an increase in lipoperoxides) in human type I diabetes and experimental diabetes.

Recent studies show that there is a redox regulation of cellular signalling and that the generation of reactive oxygen species leads to the activation of MAP-kinase pathway. This pathway induces the activation of the redox-sensitive transcription factor NF-κB that plays an important role in the regulation of gene activity.

The purpose of the present study was to test the hypothesis that free radical production in streptozotocin induced diabetes causes an activation of important cell signals as MAPKs and IκB which leads to the activation of NF-κB, and the effect of allopurinol administration (an inhibitor of the xanthine oxidase) in this process. Male Wistar rats randomly divided into 3 groups: control, diabetic and diabetic but pretreated with 32 mg/Kg of allopurinol. Western Blot analysis was used to measure ERK1/2 and IκB phosphorylation in liver homogenates.

Our results show that that Type I diabetes causes activation of MAP kinases and of IκB which is due to free radicals formed in the process. The inhibition of xanthine oxidase with allopurinol prevents this activation in the liver of the rats.

Poster - 77

Mitochondrial toxicity of beta-amyloid peptide in males but not in young females

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INTRODUCTION: Alzheimer's disease is an age-related disease of multifactorial origin. It is generally accepted that ß-amyloid peptide causes neuronal lesions that are at least in part responsible for cellular damage in this disease. There is, however, growing evidence of intracellular toxicity of ß-amyloid (βA) peptide. Its intracellular accumulation is facilitated by alpha 7 acetylcholine receptor.

AIM: The aim of this work was to examine the possibility that ß-amyloid directly increases oxidant production by mitochondria, thus activating the mitochondrial pathway of apoptosis (mediated by the release of cytochrome c). Since the incidence of Alzheimer's disease is higher in women than in men, the effect of gender of ß-amyloid peptide induced increase in oxidant production by mitochondria has also been examined.

RESULTS: We have shown that βA peptide caused an increase in the rate of oxidant production by isolated mitochondria, but only in young male mitochondria and in old female ones. Initially, these mitochondria were morphologically intact but after one hour of incubation with βA they aggregated. Reduced glutathione (GSH) prevented this aggregation. After 6 hours of incubation, βA peptide induced a released of cytochrome c from young male and old female mitochondria, but not in young female.

DISCUSSION: Our results show that the production of free radicals by mitochondria is an early phenomenon in the toxicity of βA peptide. Likewise, we found that it also caused mitochondrial aggregation and the released of cytochrome c, both of which are apoptogenic signals. The protection of young females from βA toxicity will be discussed.

Poster - 78

l-Thiazolidine-4-carboxylic acid increases survival and retards neurological and redox dysfunction in ageing mice

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l-Thiazolidine-4-carboxylic acid (*l*-4-thioproline) has *in vitro* antioxidant properties. Male mice that were supplemented with *l*-thiazolidine-4-carboxylic acid (0.2 % w/w) during their entire life showed increased 52 % and 27 % median life span and maximal life span, respectively. The body weight of mice supplemented with *l*-4-thioproline was significantly lower (15–20 %) that the weight of control mice. An inverse relation between body weight and survival was observed, in agreement with the caloric restriction concept. Supplementation with thiazolidine-4-carboxylic acid retarded the decreases in neuromotor functions (measured by the tightrope test) and in exploratory activities (measured by T maze test) associated to ageing. Tightrope success increased 70 % at 76 wk (senescent mice) and T-maze performance increased 31 % and 21 % at 52 wk (adult mice) and at 76 wk of age. Brain and liver mitochondrial enzymatic activities, NADH-cytochrome c reductase, cytochrome oxidase, and mitochondrial nitric oxide synthase, decreased by 30-61 % between 28 and 76 wk, and these losses were significantly ameliorated by *l*-4-thioproline supplementation. Brain mitochondrial enzymatic activities correlated negatively with lipid and protein oxidation products and positively with success in the behavioral tests and median lifespan.

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Poster - 79

H_2O_2 release and oxidative stress in mitochondria: Differential effects of exercise in skeletal muscle and myocardium

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In striated muscle mitochondria are the main potential source of reactive oxygen species (ROS) and, consequently, a primary target of oxidative damage. ROS seem to be implicated in the mechanism of adaptive responses to exercise and in exercise-induced tissue damage. The present study examined the effect of an acute bout of exercise on H_2O_2 liberation, antioxidant enzyme activities and oxidative stress markers levels in isolated mitochondria from skeletal muscle and myocardium. Untrained familiarized male Wistar rats (body weight 430 ± 34 g, n = 28) either remained sedentary (controls) or were exercised until exhausted (total running time 44 ± 6 min) and were sacrificed at 0-h, 2-h or 24-h post-exercise. The exercise session did not affect mitochondrial oxygen consumption supported by pyruvate/malate or succinate (plus rotenone) or palmitoyl carnitine (plus L-carnitine) as substrates. However, under the same experimental conditions, either in State 4 or in State 3, H_2O_2 release rate was lower in cardiac mitochondria from exercised (0-h and 2-h) than from control animals, whereas H_2O_2 release was higher in exercised skeletal muscle mitochondria (0-h); these differences were not observed in the presence of antimycin A. Exercise resulted in an increase in mitochondrial activities of Mn-SOD (heart and skeletal muscle) and of glutathione peroxidase (myocardium). No significant changes were detected in catalase activity or in the levels of TBARS and cardiolipin of heart or skeletal muscle mitochondria; in both tissues, the mitochondrial content in protein thiol groups was lower after the exercise session. These results suggest the existence of specific mechanisms in skeletal muscle and cardiac mitochondria that regulate contraction-induced ROS, probably related with their function in adaptive responses to exercise.

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Poster - 80

Ferrylmyoglobin *vs* metmyoglobin by infrared spectroscopy

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Heme mediated reactions have been suggested to contribute to human organ dysfunction in certain pathological conditions. In the presence of H_2O_2 metmyoglobin is oxidized to the free radical ferrylmyoglobin. Ferrylmyoglobin exert pro-oxidant effects on lipids (polyunsaturated free fatty acids, cholesterol, liposomes), proteins (thiol groups) and also induces antioxidant depletion.

Infrared spectroscopy is a useful technique for protein conformation and dynamics studies. Monitoring the IR amide I band can prove variations in protein structure. This band arises mainly from the C = O stretching vibrations and is sensitive to conformational changes. External perturbations such as temperature are commonly used to obtain a deeper insight in protein structure. More recently, Noda has proposed the use of two-dimensional correlation spectroscopy (2D-IR) to increase the amount of information obtained from the infrared spectrum.

In the present work we have compared the structure and thermal stability of both metmyoglobin and ferrylmyoglobin by monitoring changes in the study of the amide I band, concluding that although at physiological temperature secondary structure does not change, thermal unfolding is different for ferrylmyoglobin. The changes are corroborated by the use of 2D-IR.

Poster - 81

A study of 2-hydroxyestradiol-induced oxidation by infrared spectroscopy

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2-Hydroxyestradiol, the 17β-estradiol-derived metabolite, can exert both pro- and antioxidant actions depending on concentration. At physiological concentrations it acts as a pro-oxidant while at micromolar concentrations it inhibits lipid peroxidation.

Infrared spectroscopy, a useful technique in the study of protein conformation and dynamics, has been applied to study these changes. Variations in protein structure have been studied by monitoring the IR amide I band. This band arises mainly from the C = O stretching vibrations and is sensitive to conformational changes. External perturbations such as temperature are commonly used to obtain a deeper insight in protein structure by means of infrared spectroscopy. The use of two-dimensional correlation spectroscopy (2D-IR) has been proposed to increase the amount of information obtained from the infrared spectrum.

In the present work we have studied the interaction of 2-hydroxyestradiol with ferrylmyoglobin by infrared spectroscopy trying to elucidate its mechanism of action. Our results show that denaturising temperature decreases with increasing concentrations of 2-hydroxyestradiol, indicating a more loosened conformation, and that the pro/antioxidant activity is independent of tertiary structure modifications.

Poster - 82

Phenolic antioxidants content of Txakoli wines

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Phenolic compounds are naturally occurring substances in vegetables, cereals and fruits, but also in plant products such as wine, cider, beer, tea, etc. Epidemiological studies have shown that consumption of phenol-rich beverages, such as wine, is correlated with reduced coronary heart disease mortality. These protective effects are partly attributed to the polyphenolic content.

Wines constitute an excellent source of dietary polyphenols. Red wine, however, appears to exhibit biological effects that are usually not seen with white wine, which may be attributed to the large amounts of phenolic compounds, particularly phenolic acids, anthocyanins, flavanols and procyanidins which have the ability to act as antioxidants by a free radical-scavenging mechanism and metal ion chelating.

The composition of phenolics in wine depends on the type of grapes, their extraction, procedures employed for wine making and the chemical reactions that occur during the aging of wine.

In this work the evolution of the polyphenolic compounds during the vinification process in two different Txakolis, a young typical red wine of Vizcaya (Spain), is investigated. Aliquots during the vinification of the musts were taken at different times.

Two different analysis methods have been applied for the separation and quantitation of anthocyanins and the rest of polyphenols. Both of them were determined by HPLC-DAD using a C18 column and a gradient elution. Anthocyanins are quantified at 530 nm. By other hand, flavanols, phenolic acids and flavonols were quantified at 280, 320 and 370 nm respectively.

The anthocyanins are the main compounds, following the phenolic acids, flavanols and flavonols. The polyphenolic profile shows that during the maceration the phenolic content increases and when this step is finished start to decrease during fermentation and slower during maturation of wine.

Poster - 83

Identification and quantitation of polyphenolic antioxidants content in orange and apple juices

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Phenolic compounds are the class of secondary metabolites more abundant in fruits. The interest in polyphenols in medicine is related to their natural functions and physiological effects. The polyphenols are potent antioxidants, free radical scavengers and metal chelators; they inhibit lipid peroxidation and exhibit various physiological activities, including anti-inflammatory, anti-allergic, anti-carcinogenic, antihypertensive and anti-arthritic activities. Epidemiological studies have pointed out that an increase in dietary levels of these substances may be of long-term benefit to human health. Nowadays, the fruit consumption in Spain is 13 % of the intake of foods. The fruits more shrivelled are oranges and apples. The aim of this work was to develop a reversed-phase high-performance liquid chromatographic method for the simultaneous identification and quantitation of polyphenolic content presents in orange and apple juices, and in different varieties for each one of them. The samples consisted on 1 Kg of each studied fruit. The fruit was halved and juiced with a blender. The juice was mixed with 50 mL of NaF and centrifuged (6000 rpm, 15 min, 4ºC). In order to prepare each juice sample for analysis, 1 mL of centrifuged juice was pipetted into a 5 mL tube and freeze-dried. An extraction was carried out on the freeze-dried material by ultrasonic stirring with 2 mL of a methanol/water/acetic acid (30:69:1, v/v/v) mixture for 15 min at room temperature. The extract was filtered and analysed by HPLC-DAD with a C18 column and gradient elution. The polyphenols were monitorised at 280, 320, 370 and 530 nm. The results obtained allowed to notice that the main classes of polyphenols found in orange juice are flavanones and flavones specific of citrus fruit, whereas the main classes of polyphenols in apple juice are flavan-3-ols and procyanidins, dihydrochalcones, flavonols, hydroxycinnamic acids and anthocyanins.

Poster - 84

Oxidative stress biomarkers in blood and urine of hypertensive patients

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Oxidative stress is a common characteristic of the pathogenesis of hypertension. Hypertensive patients are known to present low levels of blood antioxidant enzymes and higher concentrations of oxidative stress byproducts, leading to the damage of cell macromolecules including lipids, proteins and nucleic acids (DNA and RNA).

In the present work we have examined the levels of 8-oxo-dG, MDA, and GSSG/GSH ratio in whole blood and in mononuclear cells of hypertensive patients and in healthy volunteers. In addition the yield of the damaged and mutagenic base 8-oxo-dG has been quantified in urine of the subject populations.

Blood and urine samples where obtained, stored until the performance of respective biochemical analyses following standard methodology.

Hypertensive patients present increased rates of oxidative stress as shown by the higher levels of MDA, GSSG/GSH ratio and both nuclear and mitochondrial 8-oxo-dG. The latter is also increased in the urine of hypertensive subjects as compared with healthy controls. A significant correlation exists between the levels of intracellular and urine 8-oxo-dG, suggesting the maintenance of DNA repair and the release of the damage base in hypertensive subjects.

The decrease of hypertensive values in these subjects is accompanied by the reduction of oxidative byproducts independently of the treatment used.

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