

Session 7: Oxidative Stress and Mitochondrial Function



7-01. Pathophysiological role of mitochondrial glycerophosphate dehydrogenase.

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Mitochondrial glycerophosphate dehydrogenase (mGPDH) is an important regulatory device of cell intermediary metabolism. Its activity is very high in several mammalian tissues (brown fat [1], placenta [2], beta cells of pancreas [3]) however, in most tissues its expression is highly down-regulated. In spite of many speculations about its role in various physiological (thermogenesis [4]) and pathological (hyperthyroidism [5], cancer [6]) processes, the significance of remarkable variations of the mGPDH activity in various mammalian tissues is not clear.

We have reported that mGPDH represents a new site of ROS production in brown fat [6], liver [7] and placental mitochondria [8]. In this communication we present additional data indicating: (a) that ROS production measured by Amplex Red in the presence of Rotenone, antimycin A and myxothiazol is directly connected with mGPDH catalytic function, (b) that transfer of electrons from mGPDH to CoQ has different characteristics than that from succinate dehydrogenase, (c) that correlation of triiodothyronine concentration in serum, mRNA level for mGPDH in liver homogenate, mGPDH protein content and mGPDH activity in liver mitochondria after application of a single dose of triiodothyronine to euthyroid rats indicates that this enzyme is quickly eliminated from the mitochondrial membrane when the hormonal signal disappears.

On the basis of these data potential role of mGPDH in various pathological processes is discussed.

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7-02. ROS generation by the mitochondrial respiratory chain - implication for neurodegenerative diseases.

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We have quantified the superoxide and H₂O₂ production rates of intact rat brain and skeletal muscle mitochondria under condition of oxygen saturation using *p*-hydroxyphenylacetate and Amplex red as fluorescent probes for H₂O₂ generation and hydroethidine as probe for superoxide formation. The localisation of superoxide producing sites was determined by evaluating the effects of SOD addition. In accordance with previous work [1], at comparable respiration rates and excellent functional quality of mitochondria we detected in brain mitochondria a high reversed electron flow-dependent H₂O₂ generation while the bc₁-complex-dependent H₂O₂ generation in the presence of succinate+antimycin was low. On the other hand, the reversed electron flow-dependent superoxide generation rate was small while the bc₁-complex-dependent superoxide production was considerable. In contrast, isolated skeletal muscle mitochondria showed at almost comparable reversed electron flow-dependent H₂O₂ generation more than ten-fold higher bc₁-complex-dependent superoxide and H₂O₂ generation. Our data are compatible with the following suppositions: (i) The major ROS generation site in complex I visible during reversed electron flow (very likely the FMN semiquinone moiety) is liberating superoxide predominantly to the mitochondrial matrix space. (ii) Similarly, the bc₁-complex-dependent superoxide generation site (the semiquinone at center 'o') liberates superoxide to both compartments with certain preference to the cytosolic space (in accordance with [2]). (iii) Muscle mitochondria, most likely due to their higher endogenous CoQ content, generate at comparable maximal rates of respiration considerable larger amounts of superoxide at center 'o' of complex III [3]. These findings imply a considerable role of mitochondrial complex I – dependent ROS generation for pathologies of the brain, like epilepsy and neurodegenerative diseases.

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7-03. Mitochondrial oxidative stress in genetically dyslipidemic mice.

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Atherosclerotic disease remains a leading cause of death in westernized societies, and reactive oxygen species (ROS) play a pivotal role in atherogenesis. Mitochondria are the main intracellular sites of ROS generation and are also targets for oxidative damage. Here, we show that mitochondria from atherosclerosis-prone, hypercholesterolemic LDL receptor knockout mice have oxidative phosphorylation efficiency similar to that from control mice, but have a higher net production of ROS and susceptibility to develop membrane permeability transition. Increased ROS production was observed in mitochondria isolated from several tissues, including liver, heart and brain and in intact mononuclear cells from spleen. In contrast to control mitochondria, knockout mouse mitochondria did not sustain a reduced state of matrix NADPH, the main source of antioxidant defense against ROS. Experiments in vivo showed faster liver secretion rates and de novo synthesis of triglycerides and cholesterol in knockout than in control mice, suggesting that increased lipogenesis depleted the reducing equivalents from NADPH and generated a state of oxidative stress in hypercholesterolemic knockout mice. These data

provide the first evidence of how oxidative stress is generated in LDL receptor defective cells, and could explain the increased LDL oxidation, cell death and atherogenesis seen in familiar hypercholesterolemia.

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7-04. Do mitochondrial RONS contribute to endotoxin mediated organ failure?

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An excessive production of reactive oxygen and nitrogen species (RONS) in mitochondria is considered to cause cell damage via necrosis or apoptosis. The aim of this study was to estimate the contribution of this mechanism in rats subjected to endotoxic shock. There are two common routes of endotoxin administration. Endotoxin introduced intraperitoneally (i.p.) reaches first liver and then heart. In contrast, endotoxin introduced intravenously (i.v.) reaches first heart and then liver. Therefore, the first goal of this study was to clarify whether two routes of endotoxin administration influence rat liver and rat heart mitochondria (RLM and RHM) in the same manner or not. Sprague-Dawley rats weighing 280 ± 21 g were subjected to LPS challenge 8 mg lipopolysaccharide (LPS)/kg (i.v.) and 20 mg LPS/kg (i.p.). These two doses resulted in an approx. similar mortality rate of 50 % and 30 % within 16 hours, respectively. The rats were sacrificed 16 h after LPS injection and mitochondria were prepared. Irrespectively of the route of LPS administration we observed similar changes in mitochondrial function: a significant increase in the rate of state 3 respiration in RLM, and a strong trend to a decrease in the rate of state 3 respiration in RHM. State 4 respiration rate was not influenced by LPS in both RLM and RHM. These changes were not substrate dependent (glutamate/malate vs. succinate), indicating that they do not originate from complex I or II. Thus, LPS did not have a deleterious effect on mitochondrial function. Using spin-trapping technique we detected a significant increase in RONS production in RLM, and no difference to controls in RHM. Therefore, RONS-mediated mechanisms seem to be plausible in the liver rather than in the heart. The second goal of this study was to find out whether increased RONS production in RLM has a coincidence with impaired cellular function in liver cells. We determined the release of a cytoplasmic enzyme (ALT) in blood as a marker of damaged cytoplasmic membrane of liver cells. In addition to mitochondrial function we determined enzymatic activity of cytochrome p450 in the microsomal fraction of the liver following dealkylation of 7-ethoxycoumarin. Increased mitochondrial RONS production in RLM was accompanied by increased levels of ALT in blood, suggesting the disintegration of the liver cell membranes, and by a decrease in p450 activity, suggesting a dysfunction of the endoplasmic reticulum. In contrast, mitochondrial function was not affected. We suggest that mitochondrial RONS can be involved in the oxidative damage of other subcellular organelles, but not mitochondria themselves. The latter is likely due to the strong antioxidant capacity of mitochondria.



7-05. Protection of oxidatively damaged mitochondria by novel chromanol-type antioxidants.

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Vitamin E is the most important lipophilic antioxidant protecting biomembranes from lipid peroxidation (LPO). Therefore, vitamin E and its derivatives are frequently used in the therapy or prevention of oxygen radical-induced diseases [1]. In the present study, novel chromanol-type antioxidants [2] such as the dimeric twin-chromanol, cis- and trans-oxachromanol as well as the well-known short-chain analogue of vitamin E, pentamethyl-chromanol, were tested for their antioxidative potency in rat heart mitochondria (RHM). As a prerequisite for a beneficial effect in mitochondria the tested substances should not disturb the highly sensitive function of the inner mitochondrial membrane. Bioenergetic parameters of isolated RHM, determined with the complex I substrates glutamate+malate, were not significantly changed in the presence of the highest concentration of chromanols under study (50 nmol/mg mitochondrial protein).

Exposure of RHM to an LPO-inducing system (50 μM cumene hydroperoxide plus 50 μM Fe^{2+}) significantly deteriorated their bioenergetic function. Alterations of bioenergetic parameters were partially abolished by preincubating RHM with antioxidants before adding the radical-generating system. In the lower concentration range twin-chromanol turned out to be more efficient than pentamethyl-chromanol, both being far more protective than cis- and trans-oxachromanol. Whether this protective effect was due to their antioxidative action was assessed from the measurements of protein-bound SH-groups, an indirect indicator of protein oxidation, and thiobarbituric acid-reactive substances, an indicator of LPO. For both parameters it was shown that oxidant-induced changes were partially prevented by a preincubation of RHM with chromanols.

Accumulation of increased levels of LPO products is usually accompanied or preceded by decreased α -tocopherol concentrations in lipid membranes [3]. HPLC measurements of α -tocopherol and its first stable oxidation product, tocopheryl quinone, in mitochondrial membranes have shown that α -tocopherol was rapidly consumed after the initiation of LPO. Pentamethyl-chromanol and twin-chromanol were similarly effective in preventing the decay of α -tocopherol and the increase of tocopheryl quinone levels in mitochondrial membranes. For both chromanols a concentration of 5 nmol/mg protein was required to protect endogenous α -tocopherol against oxidation, while a concentration of 1 nmol/mg protein was insufficient.

In conclusion, the new chromanol-type antioxidants, especially twin-chromanol, were able to improve bioenergetic and biochemical parameters of mitochondria exposed to oxidative stress.

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7-06. Lysosomal ROS formation.

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Ubiquinone is inhomogenously distributed in subcellular biomembranes. Apart from mitochondria where ubiquinone was demonstrated to exert bioenergetic and pathophysiological functions unusually high levels of ubiquinone were also reported to exist in Golgi vesicles and lysosomes. In lysosomes the interior differs from other organelles by the low pH value which is important not only to arrest proteins but also to ensure optimal activity of hydrolytic enzymes. Since redox-cycling of ubiquinone is associated with the acceptance and release of protons we assumed that ubiquinone is a part of a redox chain contributing to unilateral proton distribution. A similar function of ubiquinone was earlier suggested by Crane to operate in Golgi vesicles. Support for the involvement of ubiquinone in a presumed couple of redox-carriers came from our observation that almost 70 % of total lysosomal ubiquinone was in the divalently reduced state. Further reduction was seen in the presence of external NADH. Analysis of the components involved in the transfer of reducing equivalents from cytosolic NADH to ubiquinone revealed the existence of a FAD-containing NADH-dehydrogenase. The latter was found to reduce ubiquinone by means of a b-type cytochrome. Proton translocation into the interior was linked to the activity of the novel lysosomal redox chain. Oxygen was found to be the terminal electron acceptor thereby also regulating acidification of the lysosomal matrix. In contrast to mitochondrial respiration oxygen was only trivalently reduced giving rise to the release of HO[•]-radicals. The role of this novel proton-pumping redox chain and the significance of the associated ROS formation has to be elucidated.

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7-07. The preparation procedure does not influence significantly the function and morphology of liver mitochondria from control and endotoxic rats.

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Endotoxic shock is a condition in which the cardiovascular system fails to perfuse tissues adequately and cells fail to extract oxygen from blood. In each cell the mitochondria are the main oxygen sink and are thus responsible for building up an gradient which contributes to oxygen extraction from the blood. It is important, therefore, to determine adequately mitochondrial function during endotoxic shock and in other pathological states. A major problem is the isolation of mitochondria from tissues, which can be accompanied by a selective loss of a mitochondrial population with potential different size in control and experimental animals. The aim of this study was to check whether the preparation procedure exerts an influence to mitochondrial functions and morphology. Sprague Dawley rats were treated either with LPS 8 mg/kg (i.v. or i.p.). Untreated rats were used as a control. After 16 hours the livers were collected, homogenized, mitochondria were prepared and used in three experiments. The first experiment aimed at the comparison of mitochondrial respiratory parameters measured directly in liver homogenates and in isolated mitochondria using high-resolution respirometry (OROBOROS Oxygraph-2k). In these experiments we did not observe any significant difference between those parameters in homogenate and in mitochondria. This indicates that the isolated mitochondria represent the entire mitochondrial pool in liver homogenate. The second experiment aimed at the comparison of mitochondrial morphology in liver tissue and isolated mitochondria by means of electron microscopy. Staining was performed with uranyl acetate and lead citrate. The results showed that the swelling of mitochondria, typically observed in liver slices during endotoxic shock can clearly be seen in isolated mitochondria. In contrast, no swelling was observed in mitochondria isolated from control animals. This demonstrates that the morphological structure of mitochondria was not changed significantly during isolation procedure. The third experiment aimed at the comparison of the quality of mitochondrial preparations from control and LPS treated animals. We performed the analysis of typical respiratory chain markers (cyt b, cyt c1, cyt c and cyt a) in control mitochondria and mitochondria isolated from LPS treated rats by means of optical redox-difference spectroscopy using a dual-wavelength photometer. There was no significant difference between isolated mitochondria from control and LPS treated animals, indicating that there was no group-specific contamination with non-mitochondrial organelles. Together all these data suggest that the isolation of rat liver mitochondria from control and LPS treated rats does not significantly influence mitochondrial function and morphology and represent the whole mitochondrial pool in liver tissue.

7-08. Superoxide generating and cycling mechanism and its significance in energy partitioning of proton motive force in mitochondria.

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For the first time, we showed that $O_2^{\cdot-}$ and H_2O_2 generation in mitochondria were increased nonlinearly with state 4 respiration, exhibiting a non-ohmic relationship with $\Delta\Psi$ [1]. We also found that $O_2^{\cdot-}$ generated directly by a single electron leak pathway in the respiratory chain was able to induce proton leak through HO_2 transfer across the membrane into the matrix, which is formed by interaction of $O_2^{\cdot-}$ and H^+ on the outer surface of the mitochondrial inner membrane [2]. Meanwhile, we proposed a hypothetical model of cooperation of 'reactive oxygen cycle' with the Q cycle and H^+ cycle to combine both processes of electron leak and proton leak in mitochondria [1-3]. Recently, a model was proposed [4], combining our 'reactive oxygen cycle' model and the Skulachev/Garlid/Jezek fatty acid shuttling model for UCP1, of uncoupling double-loops of H^+ and $O_2^{\cdot-}$ cycling across the mitochondrial inner membrane in order to elucidate the possible function of UCPs, activated by superoxide in the mitochondrial matrix [4]. Here, we provide novel experimental evidence that $O_2^{\cdot-}$ generated in rat liver mitochondria without UCP expression was able alone to serve as an endogenous protonophore to induce H^+ leak for diverting the energy of protonmotive force (Δp) for heat production. By investigating the link between mitochondrial state 4 respiration with succinate and the generation of $O_2^{\cdot-}$ (and H_2O_2) and heat production in both euthyroid and hyperthyroid rat liver mitochondria, as well as the effects of exogenous SOD on H^+ leak, H^+ pumping and heat production in these two types of mitochondria, we revealed that (1) hyperthyroid mitochondria show an obvious increase in state 4 respiration, 71 % higher ($P < 0.01$) than euthyroid controls, with no significant increase in state 3 respiration. The values of RCR and ADP/O decreased 23 % ($P < 0.05$) and 29 % ($P < 0.05$), respectively. These results show that the alteration in respiratory chain activity of hyperthyroid mitochondria mainly lies in state 4, known to be controlled by basal proton leak of mitochondria. (2) Proton leak rate and proton pumping activity were 51 % and 120 % higher, respectively, in hyperthyroid mitochondria than in euthyroid controls. Addition of SOD decreased the proton leak by 45 % for euthyroid and 39 % for hyperthyroid mitochondria. However, SOD could give a further increase in proton pumping activity (by 45 % in euthyroid and 44 % in hyperthyroid mitochondria). The effects of SOD on both reactions exhibited a dose dependent and saturation mode, indicating that $O_2^{\cdot-}$ are involved in the process of H^+ translocation across the inner mitochondrial membrane. (3) Direct assay of $O_2^{\cdot-}$ during state 4 respiration in mitochondria revealed 40 % higher concentrations in mitochondria from hyperthyroid rat liver than in euthyroid controls. Both CCCP and nigericin depressed significantly the generation of $O_2^{\cdot-}$ and H_2O_2 . In accordance with these observations the reduction of cyt c showed more increase in hyperthyroid than euthyroid mitochondria. (4) The heat production by succinate oxidation in hyperthyroid mitochondria measured by microcalorimetry was $13.0 \cdot 10^{-2}$ J/mg protein, which is 70 % higher than that of euthyroid mitochondria ($7.7 \cdot 10^{-2}$ J/mg protein). SOD reduced the 'extra' elevated heat production of hyperthyroid mitochondria to $7.6 \cdot 10^{-2}$ J/mg protein, which equals the value observed in euthyroid controls. The elevation of average heat production in hyperthyroid mitochondria is consistent with the increase in state 4 respiration (71 % higher), and with depressed average heat production (73 % lower), as well as depressed H^+ leak rate and increased proton pumping activity by SOD. These results confirm that the stimulation mechanism of proton leak and heat production in hyperthyroid mitochondria is due to $O_2^{\cdot-}$ induced H^+ leak in association with mitochondrial state 4 respiration, and $O_2^{\cdot-}$ formed in Q cycle could be served as an endogenous protonophore for the H^+ leak to dissipate the energy of Δp , as in the 'reactive oxygen cycle' model [1-3].

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7-09. Mitochondrial superoxide production is inversely proportional to complex I activity in human complex I deficiency.

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Respiratory chain dysfunction lies at the basis of severe clinical syndromes, presenting either at birth or in early childhood, especially affecting organs and tissues with a high-energy demand, including brain, heart and skeletal muscle. Among these disorders, isolated complex I deficiency (OMIM 252010) is the most frequently encountered enzyme defect. Structurally, complex I (NADH:ubiquinone oxidoreductase; E.C. 1.6.5.3) consists of 46 subunits, seven of which are encoded by the mitochondrial DNA and the remainder by the nuclear genome. In addition to defects in the mitochondrial DNA, mutations in nuclear genes (NDUFV1, NDUFS1, NDUFS2, NDUFS3, NDUFS4, NDUFS6, NDUFS7, NDUFS8) have been shown to be associated with isolated human complex I deficiency. In order to enhance our understanding of the pathophysiology of disorders of the human oxidative phosphorylation (OXPHOS) system, we study genetically characterized patient skin fibroblasts. A previous study [1] revealed an increased production of superoxide and enhanced lipid peroxidation in human skin fibroblasts chronically treated with the complex I inhibitor rotenone. Here we determined whether enhanced superoxide generation was associated with complex I deficiency in a collection of 24 pediatric patients. To this end we applied videomicroscopy to measure the superoxide-induced conversion of hydroethidine (HET) into ethidium (Et) in living cells. Superoxide production was similar in five control fibroblast cell lines of different genetic origin and passage number, indicating that these factors are not major contributors to superoxide production in our assay. Analysis of patient fibroblasts displayed a significantly increased rate of superoxide production relative to control. Strikingly, this rate was inversely proportional to the residual enzymatic complex I but unrelated to complex III activity. This supports the idea that the superoxide detected, originates from complex I. Using Blue Native gel electrophoresis on CI deficient fibroblasts, we show that residual CI activity is linearly correlated to the amount of CI-39 kDa protein in fully assembled CI. Since superoxide production is inversely related to CI activity, which represents fully assembled and active CI protein, these findings support the idea that increased superoxide production is caused by a smaller amount of active (but less stable?) CI and not by normal amounts of 'leaky' CI.

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7-10. Relation between free iron and mitochondrial superoxide radicals during endotoxic shock.

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Oxidative stress plays an important role in the development of multiple organ failure in sepsis and endotoxic shock. Free iron is a metabolically active metal ion that increases the effects of free radical production by enhanced hydroxyl radical generation through the Fenton reaction with hydrogen peroxide. Mitochondrial superoxide radicals (SR) are considered as the main source of hydrogen peroxide. In this study we compared the levels of free iron and mitochondrial SR in endotoxic shock induced by intraperitoneal (i.p.) or intravenous (i.v.) LPS administration. Adult male Sprague-Dawley rats were injected with lipopolysaccharide (LPS) at a dose of 8 mg/kg (i.v. or i.p.) or 20 mg/kg LPS (i.p.). 16 hours after LPS application the levels of transferrin iron in blood, free iron in tissue, and mitochondrial SR were detected by means of electron paramagnetic resonance (EPR) spectroscopy. Transferrin iron levels in blood showed a significant decrease in all groups of LPS treated rats compared to control animals, suggesting the translocation of iron in tissues, mainly into tissue ferritin. However, part of this iron can appear as free iron. The injection of 8 mg LPS/kg (i.p.) did not result in an increase either in mitochondrial SR or in free iron levels. The injection of 20 mg LPS/kg (i.p.) resulted in a significant increase in both mitochondrial SR and free iron levels, accompanied by an increased mortality rate. The challenge with 8 mg LPS/kg (i.v.), also accompanied by an increased mortality rate, resulted in a significant increase in mitochondrial SR and in a trend to increase free iron levels. Since liver is the first target tissue for i.p. injection and lung for i.v., respectively, we determined free iron levels in lung after 8 mg/kg LPS i.v. administration, but did not find any change. Our data show that increased levels of free iron and mitochondrial SR in tissue may depend on the dose and the route of LPS administration and that under certain conditions free iron may amplify the oxidative potential of mitochondrial SR.

7-11. Influence of oxidative stress on osteoblasts cultured on either titanium or polystyrene.

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Titanium is a successful biomaterial used for many applications that possesses good biocompatibility and mechanical strength. It is covered by a surface layer of titanium dioxide. Mechanical disintegration of this stable inert oxide layer leads to fast reformation of titanium dioxide (corrosion). For that, the oxidation of titanium (anodic reaction) as well as the reduction of oxygen (cathodic reaction) is necessary. During the cathodic reaction at least intermediately, reactive oxygen species (ROS) can occur, which are able to affect interactions between titanium implant and surrounding tissue (monocytes/macrophages, osteoblasts, osteoclasts, endothelial cells). The surrounding cells can be activated (firstly transiently and secondly permanently) and production of reactive oxygen species by cells themselves can be induced.

In this study, we simulated oxidative stress by hydrogen peroxide and observed changes which occurred in osteoblasts cultured on either titanium or polystyrene. We determined dose-dependent but material independent cytotoxicity, different reduced glutathione levels and activation of different enzymes of oxidative defence and intracellular signalling.

7-12. MitoSOD: a novel mitochondria-targeted superoxide dismutase mimetic.

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Superoxide ($O_2^{\bullet-}$) is the proximal reactive oxygen species (ROS) generated by the mitochondrial respiratory chain, which is the major source of ROS in the cell. It is estimated that up to 1% of respiratory chain electrons generate superoxide (the one-electron reduction product of molecular oxygen) instead of contributing to the reduction of oxygen to water. Once generated, mitochondrial superoxide can react to form various other ROS, including hydrogen peroxide, peroxyxynitrite and the hydroxyl radical; together, these ROS can cause oxidative damage to all classes of macromolecules. For this reason, mitochondrial ROS are strongly implicated in mitochondrial pathology and in the ageing process. Detoxification of mitochondrial superoxide is therefore a promising therapeutic strategy; in addition, a mitochondria-specific superoxide scavenger would be an invaluable laboratory tool.

We have developed mitoSOD, a mitochondria-targeted version of the manganese superoxide dismutase mimetic M40403 [1]. MitoSOD is directed specifically to mitochondria by the inclusion of the triphenylphosphonium moiety, a lipophilic cation that drives the mitochondrial membrane potential-dependent accumulation of covalently linked compounds within the matrix (to 100-500x the cytosolic concentration) [2]. Previously, TPP-derived compounds have been shown to accumulate within mitochondria of diverse tissues upon oral delivery to mice [3], underscoring their potential utility as therapies for disorders of mitochondrial dysfunction. In this work the antioxidant efficacy of mitoSOD *in vitro* and within isolated mitochondria was characterised. MitoSOD was shown to compete with cytochrome c for reaction with superoxide, at concentrations comparable to M40403. In addition, mitoSOD competed with NO for reaction with mitochondrial membrane-derived superoxide.

The study of *in vivo* mitochondrial superoxide production and detoxification is compromised by the dearth of robust and specific intracellular superoxide probes. Oxidation of dihydroethidium to the red-fluorescent ethidium has been widely used as a measure of intracellular superoxide; however, it is highly susceptible to oxidation in air and is therefore not specific for superoxide. In addition, it does not localise specifically to mitochondria. However, it has recently been suggested that superoxide oxidises dihydroethidium to a fluorescent product that is distinct from ethidium, and is specific for superoxide [4]. We have investigated the feasibility of an HPLC-based assay for mitochondrial superoxide within cells, using mitoSOX, a commercially-available mitochondria-targeted derivative of dihydroethidium.

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7-13. Selective inhibitory effect of tBHP on respiratory chain enzymes in isolated rat hepatocytes and protective effect of SAME.

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Oxidative stress is one of the most important mechanisms through which hepatotoxins induce cell death. Effective protection of cellular damage induced by oxidants requires better understanding of reactions involved in this process. Tert-butylhydroperoxide (tBHP) has been widely used as a model compound to mimic the effect of oxidative stress in various cell types. This organic hydroperoxide is in the cells metabolized into free radicals and induces an array of cellular dysfunctions including lipoperoxidation, oxidation of NAD(P)H and functionally important -SH groups, glutathion depletion and a number of other deleterious events leading to cell death [1,2]. The aim of this work was to characterize toxic injury of isolated rat hepatocytes induced by tBHP. We also tested the potential hepatoprotective effect of S-adenosylmethionine (SAME). In the organisms, SAME participates in various transmethylation and transsulphuration reactions.

Hepatocytes were isolated from Male Wistar rats by collagenase perfusion. Isolated cells were suspended in Krebs-Henseleit medium. For estimation of toxic injury we measured activity of respiratory Complex I and Complex II (OROBOROS Oxygraph-2k, AUT), mitochondrial membrane potential - MMP (Rho 123, JC-1), MDA (TBARS) and GSH/GSSG (HPLC).

Tert-butylhydroperoxide decreases the activity of respiratory Complex I and Complex II, MMP and GSH and increases lipoperoxidation. These changes are in proportional relation to the tBHP concentration and time of incubation. Respiratory Complex I activity is much more sensitive to the peroxidative action of tBHP than the activity of Complex II. We found that SAME has protective effect against toxic injury of isolated rat hepatocytes induced by tBHP. It seems that this effect is ascribed more to transmethylation reactions than to transsulphurations.

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7-14. Evidence for increased superoxide production in atrial fibrillation detected by ESR and HPLC based assay.

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Atrial fibrillation is the most common cardiac arrhythmia. It is associated with a 5-6 fold increase in the incidence of a stroke, due to almost exclusively to thrombus formation in the left atrial appendage. We hypothesized that this decrease in NO[•] may be due to increased superoxide (O₂^{•-}) production and oxidative destruction of NO[•]. To address this hypothesis, we induced AF in pigs using rapid atrial pacing.

We studied the reaction of O₂^{•-} with a new spin probe, 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH), for analysis of O₂^{•-} production in suspension of cardiomyocytes and in isolated heart tissue. Parallel to ESR we investigated the superoxide formation using dihydroethidium and a newly developed HPLC method [1]. After 10 min of incubation, the intracellular concentration of CMH in cells reached 18.1

%. For loading the cells with DHE we incubated them for 20 min. Intracellular $O_2^{\cdot-}$ production was measured from PEG-SOD inhibited formation of 3-methoxy-carbonyl radical (CM^{\cdot}) or from selective DHE interaction product oxyethidium (OxyEt). A 1.8-fold increase in LAA $O_2^{\cdot-}$ from 80 to 140 a.u./mg tissue/10 min was confirmed using ESR and using formation of OxyEt. Treatment of cardiomyocytes with lactate leads to 3-fold increase in $O_2^{\cdot-}$ production in LAA.

We conclude that atrial fibrillation is associated with increased $O_2^{\cdot-}$ and decreased NO^{\cdot} production in the left atrial appendage. Using new synthesized cyclic hydroxylamine CMH and DHE we detected intracellular $O_2^{\cdot-}$ production from mitochondria. High cell permeability and high reactivity with $O_2^{\cdot-}$ of CMH allow effective detection of low amounts of intra- and extracellular $O_2^{\cdot-}$. These findings we confirmed using dihydroethidium and new developed HPLC based assay.

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