

## Mitochondrial $\text{Ca}^{2+}$ transport, permeability transition and oxidative stress in cell death: implications in cardiotoxicity, neurodegeneration and dyslipidemias

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### 1. ABSTRACT

Mitochondrial  $\text{Ca}^{2+}$  transport is important in the maintenance of intracellular ion homeostasis, and also a key factor in the pathogenesis of many diseases. We discuss here the main aspects of mitochondrial  $\text{Ca}^{2+}$  transport, and how this transport is linked to changes in energy metabolism and redox state. Mitochondrial permeability transition, a consequence of excessive mitochondrial  $\text{Ca}^{2+}$  accumulation associated with oxidative stress is also discussed. Finally, our current understanding of the involvement of these mitochondrial processes in cardiac ischemia-reperfusion, neurodegeneration and dyslipidemias is presented.

### 2. INTRODUCTION

A number of new proteins and modulator metabolites have been recently detected in mitochondria, steering these organelles into the spotlight in studies concerning cell signaling, cell injury, and cell death. Understanding the molecular mechanisms of ATP synthesis, electron transfer reactions and biological roles of these newly discovered mitochondrial components has provided new insights into mitochondrial physiology. Evidence has been provided that mitochondria compromise

one of the main pathways for apoptosis in vertebrate cells (see refs. 1-6, for recent reviews) and that dysfunctional mitochondria with decreased electron transfer rates and increased production rates of  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$ , both chemical species called reactive oxygen species (ROS), are associated with neurodegenerative diseases such as Huntington's, Parkinson's and Alzheimer's diseases (7,8).

### 3. MITOCHONDRIAL FUNCTION AND $\text{Ca}^{2+}$ TRANSPORT

Mitochondria are known to be the site of important metabolic pathways such as the citric acid cycle, fatty acid beta oxidation and amino acid oxidation. These organelles are also the site of oxidative phosphorylation, which is dependent on coupling between respiration, the transmembrane proton electrochemical potential and ADP phosphorylation, as postulated by Peter Mitchell (9). This transmembrane potential is generated by pumping protons across the inner mitochondrial membrane, while electrons flow through respiratory chain complexes. Proton pumping makes the matrix slightly alkaline and strongly negatively charged relative to the intermembrane space, and provides energy for ATP synthesis. Increases in the inner membrane

permeability to protons disrupt the proton electrochemical potential and may be a key event in many mitochondrially-mediated diseases. The continuous reduction of  $\text{O}_2$  by the mitochondrial electron transport chain to build up the transmembrane electrical proton potential ( $\Delta\Psi$ ) has a side effect: ROS generation (10-14).

As a result of the inner membrane electrochemical gradient, mitochondria are capable of accumulating large quantities of cations, as long as transporters or channels are present to transport the ions into the matrix. For example, mitochondria take up  $\text{Ca}^{2+}$  ions using  $\Delta\Psi$  as driving force and through the activity of inner membrane  $\text{Ca}^{2+}$  channels, accumulating large quantities of the cation (15). This effect was described more than 40 years ago (16, 17), but for a long time mitochondria were not believed to accumulate  $\text{Ca}^{2+}$  under physiological conditions, since the affinity of the channel is significantly lower than the average intracellular  $\text{Ca}^{2+}$  concentrations (15). However, more recent studies using mitochondrially-targeted aequorin (18,19) showed that mitochondria do, in fact, have an important role in physiological intracellular  $\text{Ca}^{2+}$  homeostasis, and take up  $\text{Ca}^{2+}$  due to the existence of microenvironments with high concentrations of this ion located near these organelles. This uptake is necessary to control the activities of key intramitochondrial enzymes, including pyruvate, isocitrate and  $\alpha$ -ketoglutarate dehydrogenases (20). In addition to this role in intracellular signaling, mitochondria also act as high capacity intracellular  $\text{Ca}^{2+}$  stores under pathological conditions (15). High uptake of  $\text{Ca}^{2+}$  by mitochondria can lead to the non-selective inner membrane permeabilization known as mitochondrial permeability transition (MPT).

$\text{Ca}^{2+}$  uptake into mitochondria occurs mostly due to ion fluxes through a selective ruthenium red-sensitive inner membrane  $\text{Ca}^{2+}$  channel (15,21). In addition,  $\text{Ca}^{2+}$  uptake through a mitochondrial ryanodine receptor has been reported (22). Initially,  $\text{Ca}^{2+}$  uptake by mitochondria was thought to occur exclusively in vertebrates. Later it was observed that plants, trypanosomes and other primitive eukaryotes share this same property with some specific features (23). Based on kinetic studies, Gunter's group identified two modes of  $\text{Ca}^{2+}$  uptake: standard and rapid (24), but it is not known if these forms of uptake are promoted by distinct molecular components or different activities of the same channel.  $\text{Ca}^{2+}$  efflux from the mitochondrial matrix occurs through  $\text{Ca}^{2+}/\text{Na}^+$  and  $\text{Ca}^{2+}/\text{H}^+$  exchangers.  $\text{Ca}^{2+}/\text{Na}^+$  exchange is dominant in heart, brain, skeletal muscle, and brown fat, while  $\text{Ca}^{2+}/\text{H}^+$  exchange is dominant in liver, kidney, lung and smooth muscle (25).

Evidence has been provided that alterations in mitochondrial  $\text{Ca}^{2+}$  homeostasis are linked to cellular oxidative stress and may be causes of cell death under a variety of pathological conditions. We will discuss here the mechanisms in which  $\text{Ca}^{2+}$  homeostasis and oxidative stress are linked, and present a few pathological conditions in which mitochondrial ion transport and redox state determine cell survival and death.

#### **4. MITOCHONDRIAL REACTIVE OXYGEN SPECIES GENERATION AND OXIDATIVE STRESS**

The generation of ROS by mitochondria is a continuous and physiological event. Depending on the tissue, respiratory conditions and the substrates used, 0.02 - 2% of the total mitochondrial  $\text{O}_2$  consumption results in the generation of the superoxide anion ( $\text{O}_2^-$ ) *in vitro* (10,26,27). The process is due to the monoelectronic reduction of  $\text{O}_2$  at NADH:coenzyme Q, succinate:coenzyme Q and coenzyme  $\text{QH}_2$ :cytochrome c oxidoreductases (complexes I, II and III) of the respiratory chain (10-14). These respiratory complexes are located in the inner mitochondrial membrane and  $\text{O}_2^-$  production occurs at the matrix side in respiratory complexes I and II and at the matrix and intermembrane space in respiratory complex III (11,12). There is also recent evidence that  $\text{O}_2^-$  generation is promoted by matrix-soluble dehydrogenases such as pyruvate and  $\alpha$ -ketoglutarate dehydrogenases (27,28).

The free radical  $\text{O}_2^-$  presents moderate chemical reactivity in aqueous solutions, and can suffer many different reactions within mitochondria, generating other products of the partial reduction of  $\text{O}_2$ , such as  $\text{H}_2\text{O}_2$  and hydroxyl radical ( $\text{HO}^\cdot$ ). Superoxide dismutases, present both in the mitochondrial matrix (Mn-SOD) (29,30) and in the intermembrane space (Cu,Zn-SOD) (31,32), catalyze the generation of  $\text{H}_2\text{O}_2$  from  $\text{O}_2^-$  dismutation.  $\text{H}_2\text{O}_2$  is a more stable and membrane-permeable chemical species, which can be removed by different enzymes with peroxidase activity. Alternatively,  $\text{H}_2\text{O}_2$  can generate  $\text{HO}^\cdot$ , the highly oxidative and cytotoxic ROS, through reductive homolytic cleavage. Most of the  $\text{HO}^\cdot$  generated *in vivo* probably comes from the iron-dependent breakdown of  $\text{H}_2\text{O}_2$ , via Fenton's reaction ( $\text{H}_2\text{O}_2 + \text{Fe}^{2+} \Rightarrow \text{HO}^\cdot + \text{HO}^- + \text{Fe}^{3+}$ ) (33).

Glutathione peroxidase (GPx) (34) was the first thiol antioxidant enzyme characterized in mitochondria (35). GPx removes  $\text{H}_2\text{O}_2$  at the expense of reduced glutathione (GSH), generating oxidized glutathione (GSSG). GSSG is then reduced by glutathione reductase, using NADPH as an electron source. The generated  $\text{NADP}^+$  is reduced by electrons derived from NADH by mitochondrial  $\text{NAD(P)}^+$  transhydrogenase (36). Cytoplasmic redox state, pyruvate dehydrogenase and citric acid cycle activity, respiratory rates and high-energy phosphate levels determine mitochondrial NADH levels, providing an important link between energy metabolism and mitochondrial ROS removal.

Mitochondrial  $\text{H}_2\text{O}_2$  removal is also conducted by catalase (described to date only in heart mitochondria; 37) and thioredoxin peroxidase (TPx), another thiol-dependent antioxidant (38). TPx acts similarly to GPx, but using reduced thioredoxin as a substrate. Thioredoxin reductase then recovers oxidized thioredoxin, using NADPH as an electron source. TPx is abundant in mitochondria chronically submitted to oxidative stress, such as those in the adrenal cortex (39) and is able to inhibit ROS-mediated apoptotic cell death (40). We have also showed that

exogenous TPx added to isolated mitochondria (41, 42) and endogenous cytosolic and mitochondrial *Saccharomyces cerevisiae* TPx (43) protect mitochondria against protein thiol oxidation and inner membrane permeabilization.

Mitochondria also generate the reactive nitrogen species (RNS) nitric oxide (NO), a relatively stable and membrane permeable free radical involved in many signaling pathways (see ref. 44 for review). The existence of a mitochondrial NO synthase (mtNOS) was first suggested by immunohistochemical studies that found that anti-NOS antibodies stained mitochondria (45-47). Later, the biochemical aspects of mtNOS were extensively characterized (48-50), although there is still considerable debate as to the molecular identity of mtNOS (51-53). Interestingly, mtNOS is activated by  $\text{Ca}^{2+}$  (48, 54), as observed with other NOS, providing another link between mitochondrial  $\text{Ca}^{2+}$  transport and redox state.

The main role of NO is believed to be the regulation of electron transfer, since NO reversibly inhibits cytochrome oxidase, the terminal oxidase of the electron transfer chain (51-53). As a result of this inhibition, ATP synthesis should decrease, local  $\text{O}_2$  tensions rise and  $\text{H}_2\text{O}_2$  release enhanced, effects that are not necessarily deleterious. For example, in fireflies, the resulting increase in local  $\text{O}_2$  and  $\text{H}_2\text{O}_2$  levels regulates light production (55). Another important role for NO is to regulate mitochondrial biogenesis in a manner dependent on cGMP (56). Furthermore, there is emerging evidence that the S-nitrosylation by NO of various mitochondrial proteins has an important role in intracellular signaling processes (57,58).

Because mitochondria generate both NO and  $\text{O}_2^-$ , it is expected that these two species would react generating peroxynitrite, as reported by several groups (59-63). Peroxynitrite is a highly reactive RNS, which promotes tyrosine nitration and S-nitrosation, reacting with electron transfer components and glutathione (63). In addition, peroxynitrite can react with  $\text{CO}_2$ , present abundantly in the intracellular environment, generating the highly reactive carbonate radical (64).

Mitochondrially-generated ROS and RNS can oxidize macromolecules both in mitochondria themselves and from other intracellular locations. Indeed, lipid peroxidation and DNA damage by mitochondrially-generated ROS has been extensively documented (11, 65-68). Proteins, in particular those of the inner membrane, are primary targets for the oxidative damage induced by mitochondrially-generated ROS and RNS, because the inner membrane is extremely rich in proteins, which compose more than 80% of its dry weight. Much of the oxidative damage in mitochondrial membrane proteins involves thiol oxidation and carbonyl formation. Carbonyl formation and thiol oxidation result in mitochondrial impairment with respiratory chain inhibition (69,70) and non-selective inner membrane permeabilization due to MPT (11,71,72).

## 5. MITOCHONDRIAL PERMEABILITY TRANSITION

MPT is a non-selective permeabilization of the inner mitochondrial membrane typically promoted by oxidative stress and the accumulation of excessive quantities of  $\text{Ca}^{2+}$  ions, in a process that is stimulated by a variety of compounds or conditions (25,71-74). Inner membrane permeabilization caused by MPT results in loss of matrix components, impairment of mitochondrial function and substantial mitochondrial swelling, with consequent outer membrane rupture and release of intermembrane space proteins (4,71-75). As a result, MPT actively participates in events that initiate either necrotic or apoptotic cell death.

Despite extensive research, the nature of the membrane alterations that lead to MPT still remains a debated matter. MPT clearly involves membrane proteins, almost certainly a group of modified, misfolded and assembled inner membrane components (72,76). Possible components of the MPT pore include the adenine nucleotide translocator, VDAC, cyclophilin D, hexokinase, creatine kinase and the benzodiazepine receptor (77-79).

MPT is prevented by thiol reductants such as dithiothreitol (80), while thiol oxidants promote MPT, indicating that the protein modifications that induce MPT involve thiol oxidation (80-82). Interestingly, thiol cross linkage seems to be important for these conformational changes, since dithiol reagents promote MPT and cross-linked inner membrane proteins can be observed after MPT (71,80).

We observed that a wide variety of antioxidants protect against MPT caused by distinct conditions, suggesting that this process is a result of mitochondrial oxidative stress leading to thiol oxidation (41, 80, 83-86). Further evidence that MPT was caused by ROS was provided by the discovery that this process could be promoted through the addition of exogenous sources of ROS (87,88) and RNS (89).

The link between  $\text{Ca}^{2+}$ , a necessary trigger for MPT, and enhanced ROS may be related to changes in lipid organization of the inner mitochondrial membrane promoted by interactions with this ion (90, 91). Grijalba et al. (91) reported that the binding of  $\text{Ca}^{2+}$  to the inner face of the inner mitochondrial membrane leads to the formation of cardiolipin patches. These changes in lipid organization lead to enhanced ROS release by the mitochondrial respiratory chain, promoting inner membrane thiol oxidation and MPT. In addition,  $\text{Ca}^{2+}$  ions may modulate directly the open-closed state of the MPT pore, as indicated by experiments showing that  $\text{Ca}^{2+}$  is necessary even after thiol oxidation already occurred (92-94).

## 6. MITOCHONDRIA AND CELL DEATH

Because of their central role in energy metabolism, mitochondrial function is essential to maintain cellular integrity. Indeed, many conditions which lead to

mitochondrial damage with functional impairment such as inner membrane lipid peroxidation or MPT cause necrotic cell death (11,73,74,95,96).

In addition to causing necrosis, mitochondrial membrane permeabilization can lead to apoptosis, since these organelles contain proteins involved in this process such as cytochrome c, the apoptosis inducing factor and pro-caspases (75,97-99). The mitochondrial, or intrinsic apoptotic pathway can act independently of the cell surface death receptors-mediated extrinsic apoptosis pathway to lead to cell death, or may act as an amplifying step within the extrinsic pathway (100).

Mitochondrially-mediated apoptosis is initiated when proteins participating in this process are released from the organelle into the cytosol. Since apoptogenic mitochondrial proteins are located in the intermembrane space, this release usually involves only outer membrane permeabilization, a situation which has the clear advantage of maintaining inner membrane integrity and, thus, oxidative phosphorylation (74). Outer mitochondrial membrane permeabilization is a regulated process mediated by pro-apoptotic Bcl-2 family proteins including Bax, Bid and Bad (101-103).

In addition, mitochondria can also release apoptogenic factors after their inner membranes are damaged or permeabilized by MPT, leading to matrix swelling and outer membrane rupture (73-75,95). This form of apoptosis occurs when cells are damaged or stressed in some manner that induces MPT, and thus is often seen in association with necrotic cell death. Under these conditions, the determinant factor leading toward necrosis or apoptosis will be the ability to retain physiological intracellular ATP levels (6,74,104).

### 7. MITOCHONDRIA IN TISSUE DAMAGE FOLLOWING CARDIAC ISCHEMIA-REPERFUSION

Tissue damage caused by heart attack involves a lack of adequate  $\text{O}_2$  and nutrient availability for the cardiac tissue (ischemia), followed by a return of  $\text{O}_2$  and nutrients (reperfusion). The changes in energy metabolism and redox state that occur during ischemia-reperfusion include a set of conditions that typically lead to MPT: decreased ATP levels, increases in cytosolic phosphate and  $\text{Ca}^{2+}$  levels and augmented ROS release, which are secondary to abrupt increases in  $\text{O}_2$  tensions (15,73). Indeed, MPT has been clearly demonstrated to participate in tissue damage occurring during cardiac reperfusion following ischemia by studies using radioactive deoxyglucose to measure mitochondrial swelling within intact hearts. Furthermore, tissue damage under these conditions is prevented by MPT inhibitor cyclosporin A (105). In addition, cardiac damage promoted by ischemia-reperfusion can be significantly decreased by the presence of antioxidants during reperfusion, confirming that oxidative stress is a cause of tissue damage under these conditions (106).

Cardiac damage promoted by ischemia-reperfusion can also be prevented by ischemic preconditioning, or a series of short ischemic periods preceding ischemia (107). In addition to ischemic preconditioning, cardiac tissues can also be preconditioned by mild treatments with normally damaging compounds such as  $\text{H}_2\text{O}_2$  and  $\text{Ca}^{2+}$  (108,109). These studies, in addition to experiments using selective inhibitors of signaling processes, indicate that the mechanisms through which ischemic preconditioning leads to cardioprotection are complex, and involve signaling by kinases, ROS, RNS and changes in mitochondrial  $\text{Ca}^{2+}$  and  $\text{K}^+$  homeostasis (110-112).

Several studies have demonstrated that preconditioning prevents MPT pore opening during reperfusion (109,113-115). The prevention of MPT by preconditioning is linked to the activation of ATP-sensitive inner mitochondrial membrane  $\text{K}^+$  channels (mitoK<sub>ATP</sub>; 112,114,116). These channels allow  $\text{K}^+$  ions to flow into the mitochondrial matrix, at rates much slower than  $\text{Ca}^{2+}$  uptake rates. The result of increased  $\text{K}^+$  uptake is a controlled increment in matrix volumes, which regulates the transport of ATP and ADP across mitochondrial membranes preserving the energy status of the tissue (112,117). In addition, mitoK<sub>ATP</sub> activity prevents excessive mitochondrial  $\text{Ca}^{2+}$  loading that occurs during ischemia-reperfusion (113,118), resulting  $\text{Ca}^{2+}$ -induced ROS release (116). Indeed, we have found that the mild mitochondrial uncoupling promoted by mitoK<sub>ATP</sub> activity is sufficient to decrease ROS release even in the absence of  $\text{Ca}^{2+}$  accumulation (119). Although this concept is at odds with data from other groups suggesting that mitoK<sub>ATP</sub> activity increases ROS release (120,121), we have recently found that these studies were based on artifactual increases in dichlorofluorescein (a ROS indicator) fluorescence caused by the mitoK<sub>ATP</sub> agonist diazoxide (122). The concomitant effects of mitoK<sub>ATP</sub> activity, decreasing mitochondrial ROS release and preventing excessive  $\text{Ca}^{2+}$  accumulation under ischemic conditions, certainly explain the prevention of MPT when mitoK<sub>ATP</sub> is activated by preconditioning or treatment with selective mitoK<sub>ATP</sub> agonists (122).

### 8. MITOCHONDRIA AND NEURONAL DAMAGE

Since neurons are highly dependent on oxidative energy metabolism, they are uniquely sensitive to changes in oxidative phosphorylation. In fact, mitochondrial dysfunction has been implicated in neural cell death associated with various disorders including stroke, and Parkinson's and Huntington's diseases (123-126). Energy metabolism defects in the central nervous system cause increases in neuronal intracellular  $\text{Ca}^{2+}$  levels, either by directly impairing  $\text{Ca}^{2+}$  removal systems or by excessive glutamate receptor activation, in a process known as excitotoxicity (127). Excitotoxicity is a central nervous system process in which an increased glutamate release in response to hypoglycemia, ischemia, or trauma, for example, results in neuronal necrosis or apoptosis (128). This glutamate-mediated neuronal cell death is promoted mainly by activation of *N*-methyl-D-aspartate (NMDA)

receptors, with  $\text{Ca}^{2+}$  and  $\text{Na}^{+}$  influx through respective channels (127,129). While  $\text{Na}^{+}$  influx into neurons is associated with cellular swelling,  $\text{Ca}^{2+}$  influx is correlated with cellular toxicity (129). Under excitotoxic conditions, mitochondria are the main organelle responsible for  $\text{Ca}^{2+}$  sequestration, an event associated with neuronal cell death, as long as inner membrane potentials are maintained by respiration or ATP hydrolysis (130). However, the mechanism through which mitochondrial  $\text{Ca}^{2+}$  overload signals to neuronal cell death is still unclear. As discussed previously in this review, increased  $\text{Ca}^{2+}$  concentrations in the mitochondrial matrix may induce MPT. In fact, neuronal death following hypoglycemia and brain ischemia is prevented by MPT inhibitors (131,132). However, the participation of MPT in excitotoxicity is controversial (133-136). One possible explanation for the lack of or limited participation of MPT in several experimental models of excitotoxicity is the presence of high levels of endogenous inhibitors of this phenomenon, such as adenine nucleotides and  $\text{Mg}^{2+}$  (137). Probably, experimental conditions with more severe energy deprivation (131,132,138), where phosphocreatine, ATP and ADP are depleted, favor the participation of MPT in neuronal cell death.

In stroke, changes in mitochondrial functions may affect tissue survival even after  $\text{O}_2$  and glucose return during reperfusion (139). Mitochondrial alterations reported prior to neuronal death after ischemia-reperfusion *in vivo* include impairment of respiratory chain complexes, inhibition of the pyruvate dehydrogenase complex, altered  $\text{Ca}^{2+}$  homeostasis, increased lipid peroxidation, MPT and the release of proapoptotic mitochondrial proteins (128). We observed that mitochondrially-generated ROS increased in isolated rat hippocampal mitochondria at 4 h and 48 h but not at 24 h of reperfusion after transient (10 min) global cerebral ischemia (140). Interestingly, brain damage after stroke and traumatic brain injury was diminished in mice overexpressing human uncoupling protein 2 (UCP-2) in the central nervous system (141). This neuroprotective effect is probably associated with mild mitochondrial uncoupling and decreased mitochondrial oxidative stress promoted by UCP-2. Moreover, expression of *Ucp2* was enhanced by a sublethal brain insult (141), indicating that UCP-2 may be important for ischemic preconditioning.

Parkinson's disease is characterized by rigidity, tremor and bradykinesia accompanied with loss of dopaminergic neurons in the substantia nigra. This disorder was linked to mitochondrial alterations, when 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) intoxication was discovered to cause this disease (142). MPTP causes complex I inhibition, also detected in idiopathic Parkinson's disease (125). Indeed, complex I inhibition by rotenone causes parkinsonism (143). The mechanism through which this inhibition causes dopaminergic neuron degeneration appears to involve changes in mitochondrial ROS release (143,144). We found that  $\text{Ca}^{2+}$  and complex I inhibition synergistically increase brain mitochondrial ROS release and neuronal death (145,146).

Recent work from our group has focused on neuronal dysfunction and death under situations of respiratory chain complex II (succinate dehydrogenase) inhibition. It is well characterized that succinate dehydrogenase inhibitors such as 3-nitropropionate and malonate can induce neurochemical, histological and clinical features of Huntington's disease (123, 128). Methylmalonate (147), or its metabolites (148), also inhibit succinate dehydrogenase. Methylmalonate accumulates during methylmalonic acidemia, a disorder of branched amino acid and odd-chain fatty acids metabolism, involving a defect in the conversion of methylmalonyl-coenzyme A to succinyl-coenzyme A (149). It is proposed that neuronal damage by respiratory complex II inhibitors involves impairment of energy metabolism and oxidative stress (123,124,128). Indeed, we found that respiratory chain complex II inhibitors and  $\text{Ca}^{2+}$  promote MPT in isolated organelles as well as in cultured PC12 cells and freshly prepared brain slices (138). Interestingly, our recent study indicates that treatment with diazoxide, an agonist of  $\text{mitoK}_{\text{ATP}}$ , can prevent death promoted by treatment with methylmalonate in PC12 cells and freshly prepared rat brain slices (A.J. Kowaltowski, E.N. Maciel, M. Fornazari & R.F. Castilho, unpublished observations).

## 9. MITOCHONDRIAL INVOLVEMENT IN DYSLIPIDEMIAS

Stroke and heart attack are commonly caused by atherosclerotic alterations of vessels promoted by dyslipidemias such as primary or secondary hypercholesterolemia and hypertriglyceridemia. Indeed, atherosclerotic disease remains a leading cause of death in western societies (150).

Recent findings have shown that mitochondrial energy metabolism is altered in some conditions of secondary dyslipidemias. For example, alterations in circulating lipid levels by hormones (151), dietary fat (152), and intravenous heparin plus lipid infusion (153) cause changes in the expression of mitochondrial uncoupling proteins, which are involved in the regulation of energy metabolism (154,155) and in the rate of ROS release by mitochondria (141, 154-156). In addition, the expression of UCPs is also altered in other metabolic disorders in which dyslipidemia may be present, such as diabetes, obesity and the metabolic syndrome (157-159). However, all the above conditions present a very complex metabolic context, where it is very difficult to discriminate the key causative(s) factor(s).

By using genetically modified mice we showed, for the first time, alterations in mitochondrial bioenergetics and redox state in primary hyperlipidemia (160,161). These mice models are very useful to study the effects of elevated plasma lipid levels *per se*, without other metabolic confounding factors. Mice overexpressing the apolipoprotein CIII develop severe hypertriglyceridemia and high plasma levels of free fatty acids (162) but exhibit normal glucose homeostasis (163,164). We showed that liver mitochondria from these mice present higher resting respiration and susceptibility to MPT (160). Interesting, the

phosphorylating respiration rates and phosphorylation efficiency (ADP/O ratio) were preserved. Accordingly, these results were not related to the activity or expression of UCPs (160). We proposed that the faster resting respiration represents a regulated adaptation to oxidize excess of free fatty acids in transgenic mice liver cells.

It is well known that primary hypercholesterolemia is an independent and sufficient condition to cause atherosclerosis. Cumulative experimental evidences from the 80's up to now have reinforced the "oxidative modification hypothesis of atherogenesis" (165). It postulates that the disease is triggered by the low density lipoprotein (LDL) oxidation caused by ROS from circulating and vascular wall cells. However, it is not known where and how the oxidative stress condition is established. We found that hypercholesterolemic LDL receptor gene knockout mice have higher mitochondrial ROS production rates, associated with an increased susceptibility to MPT, in several tissues (161). In addition to increased ROS production, LDL receptor knockout cells (spleen lymphocytes) present about five-fold higher intracellular Ca<sup>2+</sup> concentrations, which further contributes to higher susceptibility of MPT (G. Degasperis, B. Paim, H.C.F. Oliveira & A.E. Vercesi, unpublished observations). The increase in ROS release seems to be related to the necessity these animals have for *de novo* synthesis of triglycerides and cholesterol in each cell due to the lack of LDL uptake (161). Lipid synthesis substantially oxidizes NADPH, impairing the glutathione and thioredoxin peroxidase antioxidant systems (72). These findings provide the first evidence of how oxidative stress is generated in LDL receptor defective cells and suggest an explanation for increased LDL oxidation, cell death, and atherogenesis observed in familial hypercholesterolemia.

Treatment of hypercholesterolemia with statins (3-hydroxy-3-methylglutaryl-CoA reductase inhibitors) may also affect mitochondria, since isolated liver mitochondria from hypercholesterolemic LDL receptor knockout mice treated during 15 days with therapeutic doses of lovastatin presented a higher susceptibility to Ca<sup>2+</sup>-induced MPT (166). In addition, *in vitro* experiments showed that lovastatin (10-80 µM) induces MPT in isolated liver and muscle mitochondria, with increased inner membrane protein thiol oxidation (166). This process was dose-dependent and was more potently triggered by hydrophobic statins. The ability of statins to induce MPT may explain statin-induced apoptosis observed in cultured cells (167, 168). These effects of statins on mitochondria might lead to cell injury or death contributing to the deleterious side effects, *e.g.* myotoxicity, rhabdomyolysis and liver toxicity reported in statin-treated patients (169).

## 10. PERSPECTIVES

As a result of the discovery that mitochondria participate not only in accidental but also in apoptotic cell death, interest in the investigation of the role of these organelles in cell survival has skyrocketed. Many new and exciting discoveries have been made in the last few years,

and will certainly continue to appear in the near future. We believe that the understanding of the role of mitochondria in different metabolic syndromes (including dyslipidemias, obesity, insulin resistance and hypertension) will certainly be a focal point in these studies. Furthermore, a clearer understanding of the role of mitochondrial K<sup>+</sup> transport in cardiac tissue protection should be achieved. The exact nature of changes in ion transport, energy metabolism and redox state involved in different neurodegenerative disorders should be investigated. Finally, clear physiological and signaling roles for mitochondrial ROS and RNS should be a point of interest for new studies within the next years.

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