Free Radical Biology and Medicine 129 (2018) 1–24

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Mitochondrial calcium transport and the redox nature of the calcium-induced membrane permeability transition

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A B S T R A C T

Mitochondria possess a Ca^{2+} transport system composed of separate Ca^{2+} influx and efflux pathways. Intramitochondrial Ca^{2+} concentrations regulate oxidative phosphorylation, required for cell function and survival, and mitochondrial redox balance, that participates in a myriad of signaling and damaging pathways. The interaction between Ca^{2+} accumulation and redox imbalance regulates opening and closing of a highly regulated inner membrane pore, the membrane permeability transition pore (PTP). In this review, we discuss the regulation of the PTP by mitochondrial oxidants, reactive nitrogen species, and the interactions between these species and other PTP inducers. In addition, we discuss the involvement of mitochondrial redox imbalance and PTP in metabolic conditions such as atherogenesis, diabetes, obesity and in mtDNA stability.

1. Introduction

Mitochondria are the central hub for metabolic reactions, and the main source of cellular ATP. This organelle also comprises one of the main pathways for apoptosis and accumulates DNA mutations that may be linked to increased rates of oxidants production and several degenerative diseases. ATP is produced within mitochondria in high quantities by oxidative phosphorylation, in which nutrient oxidation, with electrons flowing through the respiratory chain, is coupled to the generation of a proton gradient across the inner mitochondrial membrane (IMM). This proton gradient (ΔP) is then used as an energy source to generate ATP. Interestingly, the negative-inside proton gradient is also the driving force for mitochondria to take up positively charged ions such as Ca^{2+}.

In this review, we describe the mechanisms involved in Ca^{2+} transport by mitochondria, focusing on Ca^{2+} effects that underlie mitochondrial redox imbalance that leads to the very complex and debated biological phenomenon termed mitochondrial membrane permeability transition (MPT) [1–4]. MPT is characterized by the Ca^{2+}-dependent opening of a non-specific pore, the permeability transition pore (PTP) in the inner mitochondrial membrane (IMM). The structure of this pore is still a matter of extensive research and has been excellently revised by experts on the topic [5–8]. The findings demonstrating that mitochondrial Ca^{2+} overload is detrimental to mitochondrial structure and function led many researchers to propose, starting in the 1970s, that this condition could be the cause of cell death via disruption of ATP production [1,9–11]. Over the following four decades, researchers utilized a variety of experimental models and methods to establish the view that, indeed, mitochondrial Ca^{2+} transport [12,13] and MPT are phenomena of physiological and pathological significance [5,7,8,14].

2. Mitochondrial calcium transport

Structural and functional effects of Ca^{2+} on isolated mitochondria, including swelling and reversible or irreversible uncoupling, have been observed since the early fifties [15–19], for more details see [20]. Indeed, achieving intact coupled mitochondria, allowing for advances in the field of mitochondrial bioenergetics, was possible only after the use of isosmotic solutions containing Ca^{2+} chelators [21]. In this respect, early investigations by Britton Chance [22] on the nature of mitochondrial reversible uncoupling promoted by moderate Ca^{2+} concentrations measured the stimulation of oxygen consumption by isolated guinea pig liver mitochondria upon sequential additions of ADP and Ca^{2+}. Using simultaneous respiration and NADH oxidation recordings, he observed that additions of either ADP or Ca^{2+} induced similar reversible activation of respiration. He proposed that Ca^{2+} could promote the release of bound ADP or stimulated ATP hydrolysis,

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https://doi.org/10.1016/j.freeradbiomed.2018.08.034
Received 31 May 2018; Received in revised form 16 August 2018; Accepted 28 August 2018
Available online 31 August 2018

which would regenerate oxidative phosphorylation, a no longer valid hypothesis.

The first mechanistic understanding of reversible mitochondrial uncoupling promoted by Ca^{2+} was reported by Deluca and Engstron in 1961 [23] and Vasington and Murphy in 1962 [24], demonstrating the ability of isolated rat kidney mitochondria to take up Ca^{2+} in a process directly dependent on respiratory energy or ATP hydrolysis-driven membrane potential ($\Delta\Psi$). Ca^{2+} uptake driven by respiration was prevented by uncouplers (dinitrophenol, dicoumarol and gramicidin) or respiratory inhibitors (cyanide, antimycin A, Amytal and azide), but not by ATP synthase inhibitor oligomycin [24]. On the other hand, Lehninger et al [25] demonstrated that Ca^{2+} uptake supported by ATP hydrolysis was inhibited by oligomycin and uncouplers, but not by respiratory inhibitors, proving it was driven by $\Delta\Psi$.

After a period of controversy [26–29], Ca^{2+} uptake was recognized to be mediated by an electrophoretic process in which Ca^{2+} influx into mitochondria carries two positive charges, thus decreasing $\Delta\Psi$. Ca^{2+} entry increases the chemical component (ΔpH) of the electrochemical potential due to charge compensation by the ejection of $2H^+$, increasing matrix alkalinization [30]. The corresponding decrease in $\Delta\Psi$ limits the influx of the cation, unless Ca^{2+} transport occurs in the presence of anions that accompany Ca^{2+} influx into mitochondria in the protonated form, neutralizing the pH gradient and thus regenerating $\Delta\Psi$ [31]. In the presence of these "permeant anions", respiration continuously restores $\Delta\Psi$ and high amounts of Ca^{2+} can be accumulated (up to 2000–3000 nmol/mg mitochondrial protein) [24]. Phosphate, that enters in the net form of H_3PO_4 through the Pi-H^+ co-transporter [32], is able to accompany Ca^{2+} entry and seems to be the most important physiological permeant anion *in situ*. In addition to favoring Ca^{2+} accumulation, phosphate reaction with free Ca^{2+} generates calcium phosphate, which precipitates in the matrix mainly in the form of hydroxyl apatite when its solubility is exceeded [33]. Indeed, x-ray diffraction analysis demonstrated the presence of amorphous $Ca_3(PO_4)_2$ granules in the mitochondrial matrix of intact animals, particularly in bone-forming tissues [31]. The current view on the role of various forms of matrix calcium phosphate in physiological or disease conditions was reviewed by Carafoli [12,34].

Given that Ca^{2+} uptake and ADP phosphorylation compete for the electrochemical gradient generated by respiration, the understanding of the energetics of Ca^{2+} transport across the inner mitochondrial membrane was relevant to clarify the coupling between respiration and ADP phosphorylation [31,35]. Interestingly, due to the lower demand of driving force for Ca^{2+} accumulation, Ca^{2+} uptake precedes ADP phosphorylation when they are simultaneously added to the reaction medium [36]. We describe next studies designed to evaluate the stoichiometric relationships between Ca^{2+} uptake, oxygen consumption, and H^+ ejection from the matrix. These studies provided the first evidence for the relationship between Ca^{2+}-induced mitochondrial redox imbalance and MPT [36].

Initially, most of the data on these stoichiometric measurements were obtained in experiments with mitochondria respiring on succinate in the presence of rotenone to block respiratory complex I [30,37]. The usual experimental protocol involved mitochondrial pre-incubation in buffered medium containing rotenone, Ca^{2+} and N-ethylmaleimide (to block the influx of endogenous contaminating phosphate, as the permeant anion). After the endogenous energy stores were exhausted and mitochondria were in a de-energized steady state, succinate was added to initiate respiration. Under these circumstances, in which no anion movements into the mitochondria take place, oxygen and H^+ measurements were performed with classical Clark and pH-sensitive glass electrodes, respectively. Changes in medium Ca^{2+} associated with Ca^{2+} uptake were monitored with a Ca^{2+}-selective electrode. Thus, the quotient of H^+ ejected per Ca^{2+} taken up gives the number of positive electric charges carried by each Ca^{2+}. The data obtained in the presence of rotenone permitted these stoichiometric relationships (H^+/Ca^{2+}, H^+/O and Ca^{2+}/O) with electrons flowing only through the two

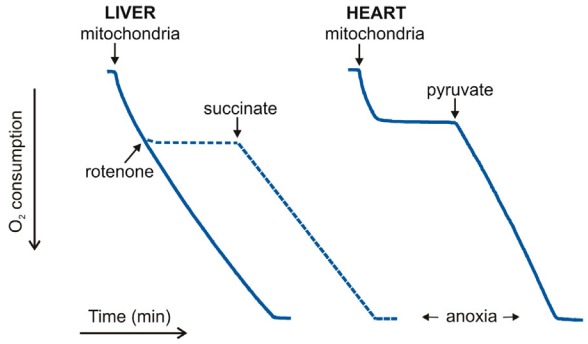

Fig. 1. Endogenous respiration by liver and heart mitochondria. Oxygen consumption was measured using a Clark-type electrode in 1.3 mL of reaction medium (30 °C), in a sealed glass cuvette equipped with a magnetic stirrer. Rat liver or heart mitochondria (1 mg/mL) were added to a reaction medium containing 125 mM sucrose, 65 mM KCl, 2 mM inorganic phosphate, 1 mM $MgCl_2$, 200 μM EDTA, 5 μM FCCP, pH 7.2. Rotenone (2 μM), 1 mM succinate or 1 mM pyruvate were added where indicated. It can be seen that liver mitochondria contained enough endogenous substrates to exhaust the total medium O_2. Therefore, de-energization of these mitochondria before O_2 exhaustion can be achieved by rotenone addition (dashed line). In contrast, the content of endogenous substrates of heart mitochondria is used after the consumption of a small fraction of medium O_2. After this, mitochondria can be re-energized by NAD-linked substrate (e.g. pyruvate) addition, allowing electrons top flow through all three energy conserving-sites of the respiratory chain.

remaining respiratory sites that conserve energy, i.e., respiratory complexes III and IV. The H^+/O ratio obtained by Lehninger's group under these conditions approached $4H^+$ per energy-conserving site per pair of electrons [30,37]. These values were criticized by others claiming that they could represent an overestimation due to leakage of electron transport from NAD-linked endogenous substrates past the rotenone block [31].

At that time, some reports suggested that heart mitochondria were not very active in Ca^{2+} transport [38]. Thus, a modified procedure permitted the isolation of tightly coupled heart mitochondria with very high ADP respiratory control ratios and high Ca^{2+} accumulation capacity and rates [36].

During aerobic incubation of these mitochondria in the absence of rotenone, Vercesi observed that they became totally depleted of endogenous substrates (in contrast to liver mitochondria) within a period of 5–10 min, after which the oxygen consumption fell to zero and the organelles were totally de-energized without the use of rotenone (see Fig. 1). This permitted, for the first time, the measurements of H^+/O and Ca^{2+}/O stoichiometry using NADH-linked substrates with electrons flowing through all three energy-conserving sites of the respiratory chain [36]. The results showed a Ca^{2+}/O ratio of 6 and an H^+/O ratio approaching 12, compatible with the energy requirements [31] of the Chemiosmotic Hypothesis of Oxidative Phosphorylation [39] and close to the value of 10 presented in the current Biochemistry textbooks. The mechanisms leading to Ca^{2+} release by mitochondria respiring in the absence of rotenone were soon uncovered by Lehninger´s group, which found that the oxidized redox state of pyridine nucleotides favored Ca^{2+} release from mitochondria [40]. We will return to this topic (Section 4).

2.1. Mitochondrial calcium uniporter (MCU)

A mitochondrial calcium uniporter (MCU) present in the inner membrane mediates the uptake of Ca^{2+} down its electrochemical gradient without coupling Ca^{2+} influx to the transport of another ion. Although this mechanism was known since the 1960's [23,24], the molecular nature of the channel was only recently identified [41]. Data indicated that the uniporter was absent in non-mammalian eukaryotic cells such as insects, plants and unicellular organisms, including yeast

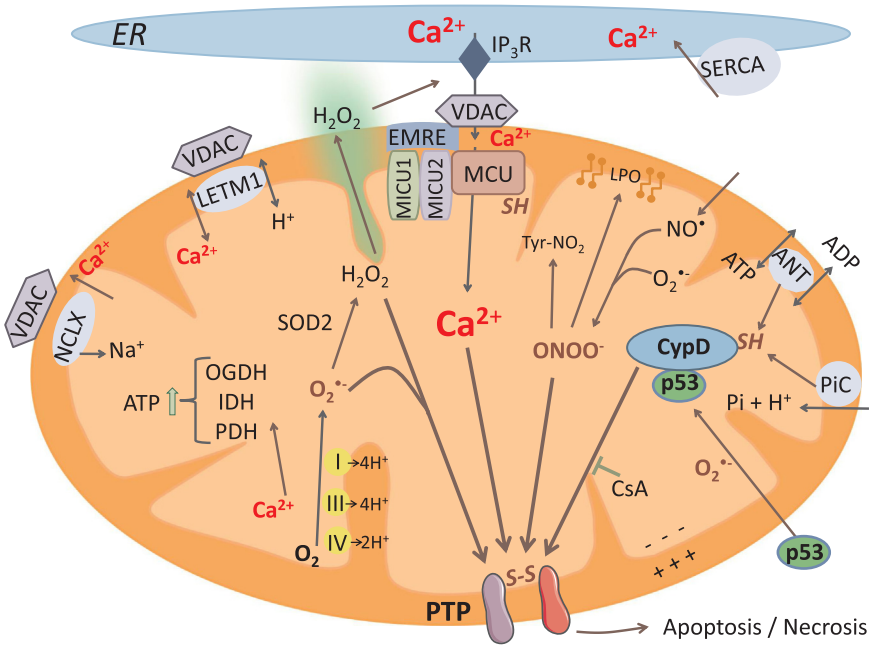

Fig. 2. Ca^{2+} transport systems and signaling effects in the mitochondrial matrix. Upon agonist signals, the ER generates sufficient [Ca^{2+}] (> 1 μM) in mitochondrial microdomains to permit fast Ca^{2+} accumulation by mitochondria. At low concentrations, the cation stimulates ATP production through the activation of matrix dehydrogenases, while mitochondrial Ca^{2+} overload promotes MPT through both stimulation of oxygen and nitrogen-derived oxidants and binding to pore membrane sites. Under mitochondrial oxidative imbalance conditions, p53 accumulates in the mitochondrial matrix and triggers MPT by physical interaction with the pore regulator cyclophilin D (CypD). Thiol crosslinking modifications occur in CypD, MCU and in structural pore proteins mediating PTP formation. The mitochondria are a target of •NO. where it can react with superoxide anion generating peroxynitrate, a short-lived oxidant that can a) react with membrane proteins forming dithiols and opening the PTP, b) generate protein 3-nitrotyrosine (Tyr-NO_2) as well as c) trigger membrane lipid peroxidation. EMRE, essential MCU regulator; ER, endoplasmic reticulum; IDH, isocitrate dehydrogenase; IP_3R, inositol 1,4,5-trisphosphate receptor; LETM1, Ca^{2+}/H^+ antiporter; LPO, lipid peroxidation; MCU, mitochondrial Ca^{2+} uniporter; MICU1, mitochondrial calcium uniporter 1; MICU2, mitochondrial calcium uniporter 2; NCLX, Ca^{2+}/Na^+ antiporter; OGDH, oxoglutarate dehydrogenase; PDH, pyruvate dehydrogenase; SERCA, sarcoplasmic ER Ca^{2+}-ATPase; VDAC, voltage-dependent anion channel. This figure was adapted from Figueira et al. [14] and based on concepts presented elsewhere [5,104,266,285,434].

[42]. However, Ca^{2+} uptake was found to occur in *Trypanosoma cruzi* mitochondria in a manner similar to that observed in mammalian cells [43,44]. Afterwards, MCU activity was described in other unicellular eukaryotes [45–49].

The identification of the molecular structure of MCU was only possible due to advances in genome sequencing and to mapping of uniporter activity among different eukaryotes (see Fig. 2). The knowledge of evolutionary conservation of MCU in vertebrates and kinetoplastids and its absence in yeast was imperative [50]. Later, RNAi screening led to the identification of the mitochondrial calcium uniporter 1 (MICU1) protein, an MCU regulator [51]. Thus, using a similar exclusion method but examining only proteins with at least two transmembrane domains that are conserved in kinetoplastids but not in yeast, one protein was identified as the MCU (NP 001028431 in *Mus musculus*) [52,53]. The two highly conserved MCU transmembrane domains are present in several eukaryotes, including trypanosomatids [50].

Mitochondrial Ca^{2+} uptake machinery is highly conserved among eukaryotes [54] and seems to be simpler in trypanosomatids compared to mammalian cells [55]. The relevance of MCU becomes more evident considering that some trypanosomatids possess orthologs of the uniporter despite the absence of functional complexes of the electron transport chain [54]. These protists are able to maintain their mitochondrial membrane potential by using the ATP synthase in the reverse mode, thus driving Ca^{2+} uptake through the MCU [54,56–58].

The uniporter structure in trypanosomes is less complex than in higher eukaryotes [59,60] but excess Ca^{2+} accumulation by mitochondria, like in mammals, stimulates oxidant generation and leads to cell death either by apoptosis [61,62] or necrosis [63].

2.2. Role of mitochondria in Ca^{2+} regulation and cellular distribution

While Ca^{2+} influx into energized mitochondria is mediated by the MCU, efflux occurs via two separate and independent pathways [64–66] (see Fig. 2). These efflux pathways promote Ca^{2+} release even when ΔΨ is too high to preclude Ca^{2+} efflux from the matrix by reversal of the MCU [67–69]. The dynamic balance between influx and efflux rates explains why Ca^{2+} distribution across the inner mitochondrial membrane does not attain the thermodynamic equilibrium distribution ratio of Ca^{2+} activities dictated by the ΔΨ, according to the Nernst equation [70], which would lead to a Ca^{2+} gradient across the inner membrane between 10^5 and 10^6 in energized mitochondria (ΔΨ 180 mV) under steady state conditions [12,13,66,71]. This means that for an extra-mitochondrial Ca^{2+} concentration of 0.4 μM, the intra-mitochondrial free Ca^{2+} would be in the range of 20 mM [66,71]. In this situation the solubility product for calcium phosphate would be exceeded, in contrast to what is observed in healthy mitochondria *in vivo* [64]. This suggests that the matrix free Ca^{2+} is much lower and not governed only by the electrophoretic uniporter mechanism. Indeed, a Ca^{2+}/Na^+ exchanger [64,68,72,73] mediates Ca^{2+} release from excitable tissue mitochondria and a Ca^{2+}/H^+ exchanger mediates Ca^{2+} release from non-excitable tissue mitochondria [21,74]. Under steady state conditions, influx and efflux pathways operate simultaneously, and Ca^{2+} cycles across the inner membrane at rates determined by the kinetic constraints of these pathways, reaching set point intramitochondrial concentrations [64,65,75]. Changes from the set point in extra-mitochondrial Ca^{2+} concentrations, such as induced by the addition of EGTA or Ca^{2+}, are followed by net Ca^{2+} release or uptake, respectively [64,65,75]. Under physiological conditions, the observed extramitochondrial set point occurs in the range of 0.3–1 μM [44,64,65,75]. The apparent Km values for matrix free Ca^{2+} are close to 9.7 μM for the Ca^{2+}/H^+ antiporter in liver mitochondria, and 5.7 μM for Ca^{2+}/Na^+ antiporter in heart mitochondria. The corresponding Vmax values were close to 4.7 and 10.8 nmol/min mg of protein in liver and heart mitochondria, respectively. Nicholls [64] estimated that, in liver mitochondria containing over 10 nmol of calcium/mg protein, the Ca^{2+} efflux pathway is close to saturation and the equilibrium value for the extra-mitochondrial free Ca^{2+} concentration is relatively insensitive to increases in matrix Ca^{2+}. Under these conditions, Ca^{2+} cycling was considered to buffer cytosolic free Ca^{2+} concentrations [65,75]. Generally, Ca^{2+} release rates values through the efflux pathways are relatively low, 100–500 times less than the Vmax for Ca^{2+} uptake via the uniporter [76–78]. Under conditions where faster Ca^{2+} release from mitochondria is necessary to attenuate Ca^{2+} overload, the opening of the PTP could be a possibility to cope with this demand [5,79].

Despite advances in the understanding of the function of the Ca^{2+} transport machinery, mitochondria were not considered a cytosolic Ca^{2+} regulator due to the low affinity of the MCU (Kd 20–70 μM) [80]. Skepticism about this issue was increased by studies with squid giant axons showing that *in situ* mitochondria were incapable of accumulating Ca^{2+} when the extra-mitochondrial concentration was below 2–3 μM, a concentration much higher than physiological (about 2 orders of magnitude) [81]. In addition, the Scarpa [81] and Somlyo groups [82] demonstrated that the levels of matrix Ca^{2+} were very low in healthy cells. Accordingly, careful homogenization and fractionation procedures to isolate mitochondria in the presence of ruthenium red or EGTA to minimize Ca^{2+} accumulation revealed values of 1–3 nmol of Ca^{2+} per mg mitochondrial protein [83]. These findings led to the general idea that mitochondria would not participate in cell Ca^{2+} homeostasis under physiological conditions [21,84], and Ca^{2+} influx was considered an artifact of *in vitro* experiments for years [13]. To worsen the mood, the discovery of the second messenger inositol trisphosphate (IP3) permitted the demonstration that Ca^{2+} mobilization in the cytosol was mediated by the endoplasmic reticulum (ER), but not mitochondria [85]. Indeed, comparative studies of cytosolic Ca^{2+} buffering ability of these two systems in digitonin-permeabilized hepatocytes indicated that the ER/sarcoplasmic reticulum (SR) presented higher Ca^{2+} affinity than mitochondria, although the capacity of mitochondria was much higher [86,87].

With respect to the role of the mitochondrial Ca^{2+} transport system in cell physiology, carefully work done by the laboratories of Denton and Hansford clarified the role of matrix Ca^{2+} in the regulation of various NADH dehydrogenases [88–92] (see Fig. 2). These groups measured the activities of matrix Ca^{2+} and Ca^{2+}-sensitive enzymes (pyruvic dehydrogenase phosphatase and α-ketoglutaric and isocitrate NADH dehydrogenases) showing that these enzymes were activated with half-maximum effects in the range of 1 μM free Ca^{2+}. These results were compatible with the regulation of the Krebs cycle activity via changes in cytosolic Ca^{2+} concentrations, thus providing reducing equivalents to the electron transport chain under increased cell demand for ATP [93,94].

Studies on Ca^{2+} transport by mitochondria regained significance upon the development of the genetically encoded chemiluminescent Ca^{2+} indicator aequorin by Pozzan´s group [95]. This Ca^{2+} indicator, specifically targeted to the mitochondrial matrix, permitted the demonstration that *in situ* mitochondria were able to take up Ca^{2+} under physiological conditions [95]. The group demonstrated that ER/SR Ca^{2+} release generates sufficient levels of Ca^{2+} concentrations in mitochondrial micro-domains to allow accumulation by mitochondria in living cells. The discrepancy between the low Ca^{2+} affinity of the MCU observed *in vitro* and the high efficiency of mitochondrial Ca^{2+} accumulation discovered by Rizzuto and Pozzan *in vivo* [96] was explained on the basis of the microheterogeneity of the citoplasmic Ca^{2+} increase under ER/SR stimulation. Under these conditions, Rizzuto and Pozzan proposed that Ca^{2+} concentration can transiently increase to 10–20 μM in the microenvironment between ER and mitochondria kinetically favoring Ca^{2+} influx. After this transient increase, extramitochondrial Ca^{2+} returns to the resting free concentrations (100 nM) in the region close to the efflux pathways and net mitochondrial Ca^{2+} efflux should occur according to the kinetic properties of the Ca^{2+}/Na^{+} or Ca^{2+}/H^{+} [96].

These data were also reproduced by other groups, strongly supporting a central role for mitochondrial Ca^{2+} transport systems in cell physiology [97–103]. In addition, these mitochondria-ER interfaces were recently shown to host nano-domains at the mitochondrial cristae containing H_2O_2 generated by the electron transport chain that act as inter-organelle communications that seem to regulate Ca^{2+} signaling and mitochondrial activities. Briefly, these nano-domains are located at the mitochondrial cristae and contain H_2O_2 generated by the electron transport chain. Changes in H_2O_2 levels in these nano-domains transfer matrix Ca^{2+} signals to the ER-mitochondrial interface [104]. The description of these H_2O_2 nano-domains added to the understanding of the cross-talk between mitochondria and the ER that mediates cell death via PTP opening [105–107]. Cyclophillin D (CypD), a well-known PTP activator [5,79], has also been proposed to modulate ER-mitochondria interactions and Ca^{2+} signals via a mechanism independent of PTP opening [108].

Interest in the role of mitochondrial Ca^{2+} transport was further stimulated when opening of the PTP was shown to initiate apoptotic cell death [109–111]. In this respect, Rottenberg and Hoek [7] proposed that mitochondrial oxidant-induced MPT has a central role in mechanisms of cell death, aging and aging related degenerative diseases such as Alzheimer´s [112], Parkinson´s [113], Huntington´s [114], amyotrophic lateral sclerosis [115], multiple sclerosis [116], diabetes mellitus [117], heart disease [118] and osteoporosis [119].

3. Mitochondrial oxidants and antioxidants

3.1. Generation and removal of oxygen-derived oxidants

As the central hub of energy metabolism and a microenvironment rich in electron transfer reactions, mitochondria are also significant sources of intracellular oxidants (reviewed in [14,120–122]). Although the production of mitochondrial oxidants is generally believed to be an unavoidable byproduct of energy metabolism, co-evolution with this process has led to the development of systems that both regulate mitochondrial oxidant production and are regulated by it. In addition, multiple cellular processes involve redox signaling in which mitochondrial oxidants participate [14,123–126]. As a result, mitochondrial oxidants are integral participants in both mitochondrial and cellular physiology and pathology.

The production of mitochondrial oxidants is typically attributed to the canonical components of the electron transport chain. Indeed, at least 4 sites within the electron transport chain have been identified as sources of oxidants in mitochondria. Notably, Complex I (including forward and reverse electron transfer), Complex II and the ubiquinone cycle within Complex III are significant, biologically-relevant, sources of oxidants (reviewed by [127]).

However, mitochondrial oxidant production is not limited to the electron transport chain. Many mitochondrial enzymes are capable of producing superoxide radicals or hydrogen peroxide as a byproduct of their primary function, including (but not limited to) glycerol-3-phosphate, pyruvate, alfa-ketoglutarate, dihydroorotate and very long chain acyl-CoA dehydrogenases, as well as the electron transfer flavoprotein [127–132]. Although often overlooked when considering mitochondrial oxidant production, the release of reactive intermediates from these enzymes can be significantly larger than that of electron transport chain components under some conditions. Furthermore, some metabolic conditions may increase oxidant production specifically at these non-electron transport chain sites [128,131].

Independently of the source of these oxidants, changes in the rates of mitochondrial oxygen consumption have an impact on oxidant production. Within the electron transport chain, increases in oxygen consumption often prevent the formation of superoxide radicals, due in part to the lower accumulation of partially reduced intermediates capable of generating superoxide radicals, and also because of lower local oxygen availability in the microenvironment (reviewed by [120]). Higher respiratory rates are promoted by lower mitochondrial inner membrane potentials, and these lower potentials further inhibit oxidant-producing processes such as reverse electron transfer (from Complex II to Complex I), adding to the prevention of oxidant formation [14,121,122].

Oxidant production by mitochondrial dehydrogenases is also indirectly regulated by oxygen consumption rates, since it is modulated by NADH and NAD^{+} levels. Production of oxidants by these enzymes is enhanced when NAD^{+} is unavailable, and therefore also inhibited by enhanced respiratory rates, which maintain the NAD pool in a more

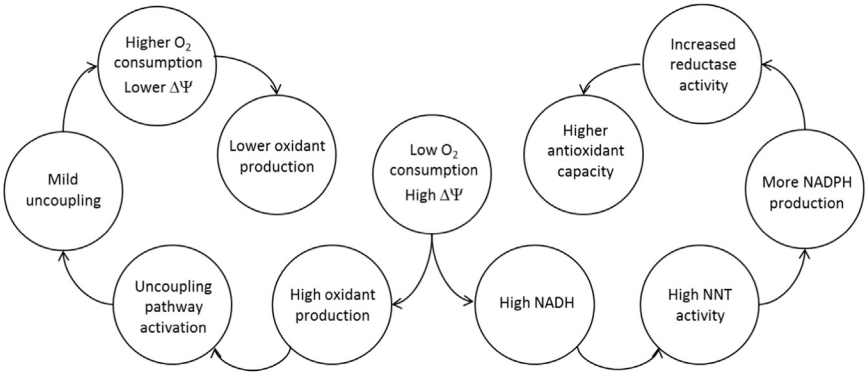

Fig. 3. Mitochondrial oxidant production and antioxidant capacity are intimately linked to electron transport rates and inner mitochondrial membrane potentials (ΔΨ). Low electron transport rates and high ΔΨ lead to higher oxidant generation at various mitochondrial sites, but also activate mitochondrial uncoupling pathways, which decrease ΔΨ, increase respiration and decrease oxidant formation. In parallel, high ΔΨ and low electron transport rates concertedly improve NNT activity, providing more NADPH within mitochondria, and thus enhancing the antioxidant and reductase capabilities of the organelle.

oxidized state [128,129,131,132]. The central importance of respiratory rate regulation is illustrated by the fact that mild mitochondrial uncoupling, increasing respiratory rates by promoting slightly higher inner membrane permeability to protons, strongly prevents oxidative damage in many key tissues [133].

Indeed, one of the primary mitochondrial strategies to control oxidant production and therefore maintain physiological redox states is by activating mitochondrial uncoupling pathways. Oxidants have been widely shown to activate these pathways, including uncoupling proteins, anion carriers and K^+ transporters [134–136]. The resulting mild uncoupling prevents oxidant production in an elegant and simple negative feedback loop [135,137] (see Fig. 3).

Other mitochondrial antioxidant strategies include the abundant presence of antioxidant enzymes. Mitochondria contain superoxide dismutases both in the matrix and in the intermembrane space, reflecting the fact that superoxide radicals are produced in both compartments, and that this radical is poorly permeable across the inner membrane [14,138,139]. Hydrogen peroxide produced by this dismutation or by the activity of mitochondrial oxidases can be removed by catalase (present in some tissues, such as heart and liver) or peroxidases utilizing thiol-containing compounds such as thioredoxin and glutathione as coenzymes [14,140–142], also present in distinct mitochondrial compartments [143,144]. These enzymes use electrons donated by thioredoxin or glutathione, which are then re-reduced by mitochondrial thioredoxin reductase and gluthathione reductase, using NADPH as the electron donor [145,146]. As a result, mitochondrial NADPH is critical in the maintenance of mitochondrial redox state (see Fig. 4).

Mitochondrial NADPH pools are physically and functionally separate from cytosolic pools, and thus completely unrelated to pentose pathway activity. Instead, mitochondrial NADPH is produced by enzymes isocitrate and glutamate dehydrogenase (which can reduce both NAD^+ and $NADP^+$, [14,147–149] and, in a more prominent manner, by the nicotinamide nucleotide transhydrogenase (NNT), which uses the inner membrane proton gradient as a driving force to transfer electrons from NADH to $NADP^+$ [150]. The NADPH pool, as a result, is maintained more reduced under high inner mitochondrial membrane potentials and plentiful NADH, the exact same conditions that favor the production of oxidants that NADPH helps remove. Thus, in addition to a pathway regulating oxidant production, an elegant system links conditions of high mitochondrial oxidant release with more efficient mitochondrial antioxidant mechanisms (see Fig. 3).

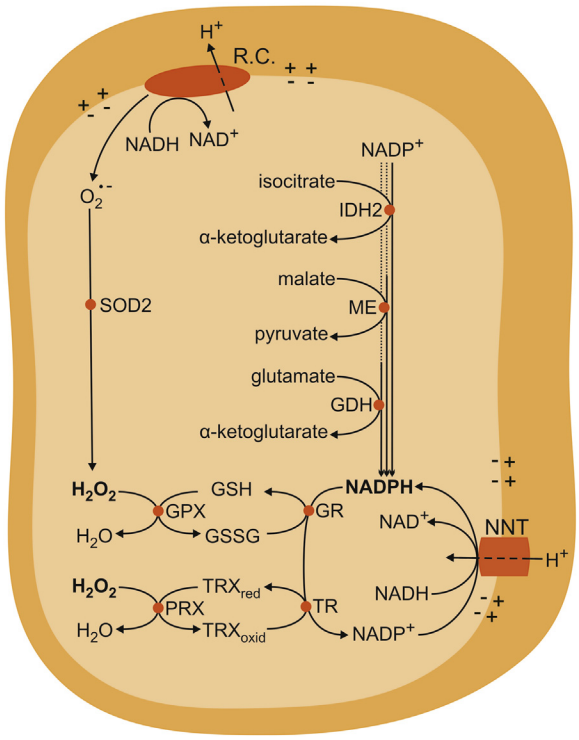

Fig. 4. Sources of NADPH and H_2O_2 metabolism in the mitochondrial matrix. $NADP^+$ is reduced to NADPH by the activity of the enzymes isocitrate dehydrogenase 2 (IDH2), malic enzymes (ME) and glutamate dehydrogenase (GDH). In addition, $NADP^+$ is also reduced by the forward reaction of the nicotinamide nucleotide transhydrogenase (NNT). Glutathione reductase (GR) and thioredoxin reductase (TR) catalyze NADPH-dependent reduction of glutathione disulfide (GSSG) and the oxidized form of thioredoxin (TRX_{oxid}), respectively. Respiratory chain (R.C.)-generated $O_2^{\bullet-}$ is dismutated to H_2O_2 via matrix superoxide dismutase (SOD2). H_2O_2 is metabolized by glutathione peroxidase (GPX) and peroxiredoxin (PRX), oxidizing glutathione (GSH) and thioredoxin (TRX_{red}), respectively.

3.2. Reactive nitrogen species (RNS) and the mitochondria

The nitric oxide radical ($^{\bullet}NO$) is a compound that is gaseous, short-lived, diffusible through membranes and endogenously produced in living organisms. This radical is widely recognized for its role as a signaling molecule in various biological processes [151] and its physiological formation occurs via two biochemical reactions, as depicted below, from the substrates L-arginine or inorganic nitrite (NO_2^-) [151–154].

L-arginine + NADPH + O_2 + H^+ → $^{\bullet}$**NO** + L-citrulline + $NADP^+$ (Reaction 1)

NO_2^- + ED_{red} + H^+ → $^{\bullet}$**NO** + ED_{oxi} + OH^- ; ED = electron donor (Reaction 2)

Reaction (1) is catalyzed by $^{\bullet}NO$ synthase (NOS) isozymes and Reaction (2) results from the nitrite reductase activities of some proteins, notably mitochondrial respiratory chain complexes, cytochrome *c* and heme deoxyglobins [154–156]. The availability of NO_2^- to fuel Reaction

(2) is warranted, because diet-acquired NO_3^- is reduced to NO_2^- by commensal bacteria in the oral cavity [153,157]. Within the gut, the acidic environment of the stomach also favors the biotransformation of NO_2^- into nitrogen oxides, thus comprising another source of $^{\bullet}NO$ that may not be only available locally but also could affect systemic pathways following absorption of nitrated molecules [158]. These interconversions between NO_3^-/NO_2^- and $^{\bullet}NO$ have been termed the nitrogen oxide cycle and are of importance for human physiology and pathophysiology [153]. More information about the function of this cycle or NOS isozymes as sources of $^{\bullet}NO$ can be found elsewhere [153,159]. Because of $^{\bullet}NO$ properties in cells (e.g. stability and diffusion) [160], this radical is available in the mitochondrial microenvironment regardless of whether a specific NOS isozyme is located in mitochondria, which may be a tissue-specific feature [161] and is also a subject of debate [152].

•NO may affect mitochondrial function and molecular integrity in different manners [151,162–166], either directly [166] or indirectly following the formation of secondary reactive species which are more oxidative [165,167]. To understand the formation of derived species, it is important to remember that mitochondria generally behave as net sources of $O_2^{\bullet-}$ in cells and that the production of this radical can be increased under some circumstances. Chemical properties predict that $O_2^{\bullet-}$ is more likely to react with $^{\bullet}NO$ ($^{\bullet}NO + O_2^{\bullet-} \rightarrow ONOO^-$) than to be dismutated into H_2O_2 by SOD under *in situ* conditions, producing the peroxynitrite anion ($ONOO^-$, pK_a 6.8) [168]. A minor fraction (~20 to 39%) of this secondary RNS is protonated (ONOOH) in a physiologically-relevant pH range (7.4–7.0) and is diffusible through membranes. Thereby downstream redox reactions are not confined to the cellular compartment of its production. $ONOO^-$, the ionized-predominant form of this chemical specie in cell pHs, can be formed in mitochondria and its high reactivity can cause oxidative damage to biomolecules and organelle dysfunction [165,167–170]. Yet the oxidant and damaging activity of $ONOO^-$ may reside in derived radicals formed by reactions with CO_2, transition metal centers and homolytic cleavage, namely: carbonate ($CO_3^{\bullet-}$), nitrogen dioxide ($^{\bullet}NO_2$) and hydroxyl ($^{\bullet}OH$) radicals [168]. For additional details on this topic, we refer the readers to a recent extensive and comprehensive review that fully covered the biochemistry of $ONOO^-$ and its downstream reactions with other biomolecules [171].

4. Mitochondrial permeability transition (MPT)

As mentioned above, evidence that Ca^{2+} promotes uncoupling, ultrastructural changes and swelling has been reported since the pioneering initial work with isolated mitochondria [12,33,111]. A large body of evidence indicates that these mitochondrial alterations are secondary to a Ca^{2+}-dependent opening of a proteinaceous megachannel, the permeability transition pore (PTP) which may exhibit different conductance states [6,172–174]. Under controlled experimental conditions the pore may not be detrimental to mitochondrial coupling and structure [175–178] and may have physiological functions such as regulation of Ca^{2+} release [173,179]. However, at the maximal conductance state, the PTP permits equilibration of solutes up to 1500 Da or even the release of small matrix proteins, eliminating all mitochondrial energy-linked functions [5,174,180,181].

The relevance of MPT was initially questioned due to the extravagant non-physiological conditions required to trigger the phenomenon in isolated mitochondria [182,183]. However, better understanding of the factors controlling opening and closing of the PTP and the demonstration that MPT inhibitors, like cyclosporin A (CsA) [184,185], prevent cell death under many pathological conditions [186,187] confirmed the participation of MPT in the pathogenesis of many diseases. These include ischemia/reperfusion, heart disease, neurodegenerative diseases, inflammation, traumatic brain injury, muscular dystrophy, drug toxicity and aging [5,7,182,188–197].

The amount of Ca^{2+} loading that triggers MPT varies with mitochondria from different origins and a series of conditions or the presence of so-called inducing agents [180,198]. Despite an enormous amount of literature data, the molecular identity of PTP components remains unresolved and controversial [5,7,172,199]. Various matrix, inner and outer membrane proteins have been suggested as PTP components, such as the adenine nucleotide transporter (ANT), the CsA-binding protein cyclophilin D (CypD), the voltage dependent anion channel (VDAC), hexokinase, aspartate-glutamate and phosphate (PiC) carriers and the spastic paraplegia 7 protein (SPG 7) [179,200–205]. Later, the structure of the PTP was proposed to be composed by the lateral stalk [206] or the c-ring [6,207–210] of the F_1F_O ATP synthase. However, recent work from John Walker and coworkers proposed that both subunits are not involved in PTP structure [211,212]. Other studies using different approaches suggest that although the PiC may have a regulatory role in PTP opening, it is not a critical component of the pore [213–215]. Ablation of SPG7, a protease that co-assembles with a homologous protein, AFG3L, and other unidentified proteins, protects against PTP opening, thus suggesting that it may have a role in PTP regulation or structure [205]. In addition, studies using inverted submitochondrial particles, mitoplasts and plant mitochondria demonstrated that some of these proteins are also not essential components of the PTP [216–219]. Accordingly, PTP opening could be monitored in mitochondria genetically deficient in ANT, VDAC or CypD, although with different properties [220–223]. For example, in CypD-deficient mitochondria, MPT requires larger Ca^{2+} loads and is not blocked by CsA [201]. In addition, in ANT-deficient mitochondria, atractyloside, an ANT inhibitor, does not promote PTP opening [221]. Interestingly, trypanosomatid mitochondria, which possess a mitochondrial CypD homolog (*TcPyD22*) to the mammalian CypD [62], have much higher resistance to PTP opening than mammalian mitochondria [224]. However, overexpression of *TcPyD22* in these parasites enhances loss of mitochondrial $\Delta\Psi$ and cell viability when H_2O_2 is present, via a mechanism sensitive to CysA [62,225]. Taken together, these results suggest that PTP may be formed with different properties in the absence of one or more components.

4.1. Mitochondrial Ca^{2+} release and MPT induced by the oxidized state of endogenous NAD(P)H: historical background

Among MPT inducers with higher biological relevance are inorganic phosphate, fatty acids, NADPH oxidants and nitric-oxide-derived species [14]. In this respect, we describe below the sequence of events demonstrating the role of NADP redox state, oxidant generation and membrane protein thiol oxidation in Ca^{2+}-dependent MPT.

The observation that rat heart mitochondria possess low endogenous substrate contents and lower ability to retain accumulated Ca^{2+} in the absence of rotenone [36] led to the design of a protocol to investigate whether stimulation of Ca^{2+} efflux from mitochondria could be regulated by the redox state of endogenous pyridine nucleotides. Ca^{2+}-loaded liver mitochondria respiring on succinate in the presence of rotenone could not retain matrix Ca^{2+} when NAD(P)H was oxidized by either oxaloacetate or acetoacetate. In contrast, the addition of the reductant β-hydroxybutyrate during the onset of Ca^{2+} release promoted $NAD(P)^+$ re-reduction followed by Ca^{2+} uptake and retention (see Fig. 5**A**). More than one cycle of Ca^{2+} release and reuptake could be repeated by alternating cycles of NAD(P)H oxidation and reduction [40] (see Fig. 5**B**). Based on these results, Lehninger proposed a working hypothesis in which Ca^{2+} release associated with NAD(P)H oxidation and a low cytosolic phosphorylation potential (ΔGp) could function as a feedback mechanism to increase the cytosolic phosphorylation potential through the stimulation of cytoplasmic catabolism by mitochondrial Ca^{2+} that was released [40].

These findings stimulated many groups to work on this topic and many laboratories reproduced these results in isolated mitochondria [226–238], intact cells [239] and perfused liver [240]. While the work with cells and organs suggested a physiological role for Ca^{2+} release

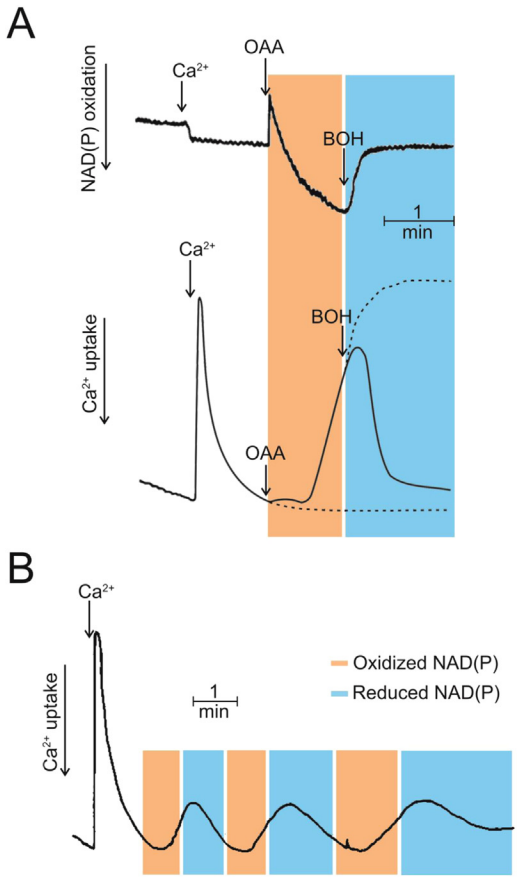

Fig. 5. Effect of matrix NAD(P) redox changes on mitochondrial Ca^{2+} transport. Panel A, NAD(P) autofluorescence and Ca^{2+} uptake were measured simultaneously in a suspension of isolated liver mitochondria. The reaction was initiated by the addition of mitochondria (4 mg) to a medium (2.0 mL) containing 65 mM KCl, 125 mM sucrose, 3 mM Hepes, 5 mM $MgCl_2$, 0.2 mM phosphate, 4 μM rotenone and 1 mM succinate, pH 7.2. Ca^{2+} (630 nmol), oxaloacetate (OAA; 0.5 mM) and β-hydroxybutyrate (BOH; 2.4 mM) were added as indicated. **Panel B**, transient release and reuptake of Ca^{2+} by isolated liver mitochondria. The reaction was initiated by the addition of mitochondria (11 mg) to a medium containing 10 mM acetate to replace phosphate. Successive additions of OAA (0.5 mM) and BOH (5 mM) promoted NAD(P) oxidation and reduction, respectively. This figure was adapted from Lehninger et al. [40].

associated with NAD(P)H oxidation, other researchers questioned the biological significance and the molecular mechanisms involved in this process [229,231,233,234]. The most significant data against the physiological role of pyridine nucleotide redox state in the regulation of cytosolic Ca^{2+} concentration via an electroneutral $2H^+/Ca^{2+}$ exchange were published by Nicholls and Brand [229]. Based on experimental data, these authors claimed that Ca^{2+} release by NAD(P)H oxidants was paralleled by uncoupling and complete collapse of the membrane potential owing to loss of mitochondrial integrity. In addition, they showed that the process was stimulated by inorganic phosphate [180]. They also provided evidence that ATP plus oligomycin, that inhibit mitochondrial disruption by Ca^{2+}, blocked these mitochondrial alterations. Furthermore, they proposed that the $2H^+/Ca^{2+}$ stoichiometry observed by Fiskum and Lehninger [241] under these conditions was consistent with the charge movements of $2H^+$ moving into the matrix to compensate for the efflux of one Ca^{2+} via reverse of the uniport. Despite the conclusions by Nicholls and Brand that NAD(P)H oxidation acts through increasing mitochondrial sensitivity to Ca^{2+}-induced damage, the interest on the topic continued owing to the increased relevance of mitochondrial dysfunction and redox imbalance associated with alterations in Ca^{2+} homeostasis in cell death [10,242–244].

Two main hypotheses were proposed to explain the molecular mechanisms involved in the process of this Ca^{2+} release from mitochondria upon NAD(P)H oxidation. **First**, Ca^{2+} release induced by NAD(P)H oxidants occurred from intact mitochondria via a route that was regulated by protein ADP-ribosylation after hydrolysis of intramitochondrial NAD^+ by a Ca^{2+}-dependent NADase, producing ADP-ribose, nicotinamide, and 5`-AMP [245]. Up to now, the participation of ADP-ribosylation in PTP opening has not been confirmed. **Second**, Ca^{2+} release occurred through nonspecific leaks caused by accumulation of free fatty acids and lysophospholipids when the activity of the inner membrane-associated phospholipase A_2 is increased by matrix Ca^{2+} and acyl-Coa:lysophospholipid acyltransferase is inhibited due to oxidation of sulfhydryl groups [246]. This hypothesis was ruled out by the same proponent group which could not demonstrate the presence of the latter enzyme in the matrix [247].

The potential importance of clarifying mechanisms underlying the correlation between pyridine nucleotide redox state and Ca^{2+}-induced effects in mitochondria led Vercesi to continue his investigations on this topic. The first challenge was to look for possible experimental conditions in which $NAD(P)^+$-induced Ca^{2+} efflux could be dissociated from swelling and uncoupling. Indeed, mitochondrial swelling was found not to be mandatory for Ca^{2+} efflux induced by the oxidized state of NAD(P) when medium sucrose was partially replaced by large molecular weight polyethylene glycol. Moreover, when the regular sucrose medium was complemented with 20 mM K-acetate instead of inorganic phosphate as the permeant anion, longer periods of $NAD(P)^+$-stimulated Ca^{2+} release occurred, and could be completely reversed before the appearance of swelling [176]. In addition, when experiments were run in the presence of ADP, Mg^{2+}, and oligomycin, the addition of ruthenium red allowed for the estimation of a net increase in the rate of $NAD(P)^+$-stimulated Ca^{2+} release from 3.6 to 9.0 nmol/min mg [177]. Most interesting, FCCP addition during the onset of Ca^{2+} efflux induced by $NAD(P)^+$ enabled the estimation of a respiratory control over 6 and similar to the control experiment, thus strongly demonstrating that this Ca^{2+} efflux could occur from tightly coupled mitochondria [177]. Another set of experiments provided evidence that **i)** ruthenium red-promoted Ca^{2+} release was specifically stimulated by $NADP^+$ and not NAD^+, and, **ii)** when the redox state of $NADP^+$ was shifted to a more oxidized state, the steady-state level of external Ca^{2+} increased from 0.6 to 0.9 μM, while $\Delta\Psi$ decreased from 100 to 80 mV, until NADP was fully reduced again. Although these results provided evidence that Ca^{2+} release under controlled experimental conditions could be dissociated from mitochondrial damage, it could not be dissociated from $\Delta\Psi$ decrease. It should be emphasized, however, that the redox state of $NADP^+$ was manipulated through the combination of oxidant/reductants plus different mitochondrial energy states by using different concentrations of the electron donor TMPD in combination with ascorbate [178]. Although these experimental conditions were far from physiological, the demonstration of NADP specificity for Ca^{2+} release was in agreement with previous data [230] and suggested the participation of the nicotinamide nucleotide transhydrogenase (NNT) in the process, a topic developed in detail below. Since the discovery that CsA is a potent inhibitor of either PTP or $NAD(P)^+$-induced Ca^{2+} efflux [185], NAD(P)H oxidants began to be included among MPT inducers.

4.2. $NADP^+$-induced Ca^{2+} efflux can occur in the presence of physiological Mg^{2+} and ATP concentrations

The inhibition of $NADP^+$-induced Ca^{2+} efflux by ATP [229] argued against both its occurrence under *in situ* conditions and its biological importance. In this context, Bernardes and coworkers [175] analyzed Ca^{2+} flux and the redox state of mitochondrial pyridine nucleotides in the presence of *t*-butylhydroperoxide (*t*-BOOH) or acetoacetate when isolated liver mitochondria were exposed to 20 nmol Ca^{2+} per mg protein and physiological concentrations of ATP and Mg^{2+}. Transient

NADPH oxidation during an interval in which peroxide was metabolized at the expense of NADPH reducing equivalents occurred in parallel to a transient increase in extra-mitochondrial Ca^{2+} concentrations. This transient increase in extra-mitochondrial Ca^{2+} concentrations occurred provided acetate, instead of phosphate, was used as the permeant anion. These events were associated with both a small transient decrease in ΔΨ (20 mV) and a small amplitude mitochondrial swelling. Acetoacetate failed to oxidize NADPH and to promote net Ca^{2+} release, in agreement with the previous findings [178] that acetoacetate does not oxidize NADPH at high membrane potentials, and therefore failed to stimulate net Ca^{2+} efflux from mitochondria [229]. After a cycle of net Ca^{2+} release and re-uptake in the presence of ATP and Mg^{2+}, the degree of mitochondrial coupling was largely preserved despite the small swelling. Further ΔΨ measurements in the presence of ruthenium red or EGTA, added after a cycle of NADPH oxidation and Ca^{2+} release, lead to the proposal that the measured ΔΨ decrease was caused by at least three different energy-dissipating processes: ***a***) faster Ca^{2+} cycling, ***b***) a ΔpH-dependent transhydrogenase reaction transferring reducing equivalents from NADH to $NADP^+$ and, ***c***) a transient non-specific increase in membrane permeability, increasing the conductance to H^+, Ca^{2+} and even to medium sucrose, that explained the parallel mitochondrial swelling. The latter component of ΔΨ decrease could be augmented by either increases in Ca^{2+} load or by omitting ATP from the reaction medium. Again, these results showing ΔΨ decrease do not support the idea that $NAD(P)^+$-induced Ca^{2+} release occurs through an electroneutral $Ca^{2+}/2H^+$ exchange [241] even in the presence of physiological ATP and Mg^{2+} concentrations. However, the reversibility of $NADP^+$ and Ca^{2+}-induced release, notwithstanding its molecular nature, opened avenues for the investigation of multiple forms and specificity of pore conductance [174,248–250].

Based on these results, we revisited data showing opening of PTP without loss of respiratory control by high Ca^{2+} concentrations and acetoacetate in mitochondria energized by succinate in the presence of ADP, Mg^{2+} and oligomycin [177]. In this new study [174] we verified that FCCP addition triggered a fast oxidation of NADPH that shifted a low to a high conductance state of acetoacetate-induced PTP. This was illustrated by fast and high amplitude mitochondrial swelling. The interpretation is in agreement with the data indicating that NADPH oxidation by acetoacetate does not occur at high ΔΨ. Large amplitude swelling started after a full oxidation of NADPH [174,178].

4.3. MPT is promoted by thiol oxidation

Early studies indicated that mitochondrial swelling induced by Ca^{2+} was mediated by membrane thiol oxidation [251–253] (see Fig. 2). In this respect, evidence was provided that MPT was prevented by thiol reductants [216,237,254–258], while thiol oxidants such as diamide, phenylarsine oxide and 4,4P-diisothiocyanato-stilbene-2,2P-disulfonic acid (DIDS) promoted MPT [256,259–261]. In addition, the similar inhibition of *t*-BOOH or diamide-induced Ca^{2+} efflux by dithiothreitol [251,252] led to the hypothesis that $NADP^+$-induced Ca^{2+} release occurred via membrane sulfhydryl-disulfide transitions [262]. Indeed, Fagian et al [216] demonstrated that membrane permeabilization induced by Ca^{2+} plus diamide in heart submitochondrial particles was caused by protein cross-linking formation due to oxidation of critical sulfhydryl groups. The presence of these large molecular weight protein aggregates was documented using sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel electrophoresis of solubilized membrane proteins. Likewise, the generation of protein aggregates was confirmed by SDS-PAGE in mitoplasts exposed to Ca^{2+} and diamide or *t*-BOOH [256,257,263]. Pre-treatment of heart submitochondrial particles or mitoplasts with b-mercaptoethanol, a thiol reductant, was found to reverse both Ca^{2+}- induced permeabilization and generation of protein aggregates. Most interesting, treatment of the gels with b-mercaptoethanol before staining with silver nitrate eliminated the protein aggregates and recovered most of the control protein bands. These results strongly support the current propositions that PTP is not a molecularly defined channel, but rather a permeability transition caused by thiol cross-linking of various mitochondrial proteins [5,203,216,264–266].

The possible role of CypD as a thiol redox sensor in the mechanism of protein-protein interactions is also in line with the data by Fagian et al. [216]. In this respect, CypD S-nitrosylation at Cys203 prevents PTP generation due to inhibition of CypD interactions with proteins involved in PTP formation [267]. In contrast, cross-linking between ANT Cys56 and Cys159 induced by oxidative imbalance or thiol reagents enhances CypD-ANT interaction and PTP opening [268] (see Fig. 2). The implications of these CypD modifications on PTP activity as well as the different roles of CypD in cellular physiology and mitochondrial bioenergetics are reviewed in more details by Javadov et al. [5].

A finding of significant importance relative to CypD participation in PTP opening was the identification of a p53-CypD complex in the event of mouse brain ischemia/reperfusion injury [269]. The authors provided evidence that p53 moves from the cytosol to the mitochondrial matrix in response to oxidative imbalance that triggers PTP opening by interacting with CypD. It has also been shown that CsA pretreatment or ablation of p53 prevented both p53-CypD complex formation and stroke injury. Further work [270–272] supports these findings as well as a role for the p53-CypD complex in the mechanisms of PTP-induced cell death.

4.4. Oxidant participation in MPT

The evidence that MPT induced by Ca^{2+} was stimulated by thiol oxidation [203,216,256,257] and by exogenous oxidant generating systems such as xanthine/xanthine oxidase [273,274], menadione [275], nitrofurantoin [276] and 5-aminolevulinic acid [257,277] and depletion of mitochondrial NADPH [178,259] and, in contrast, protected by a variety of antioxidants [256,258,278–281] or the absence of molecular oxygen [263] led the Vercesi group to directly test the hypothesis that MPT was caused by mitochondrially-generated oxidants. Indeed, strong evidence for the participation of H_2O_2 was obtained in studies demonstrating protection against MPT by catalase [174,256,263,278,282], peroxiredoxin [279] and *o*-phenanthroline [263].

While pore “inducing agents” such as Pi [79,180], oxidants and thiol cross-linkers have a facilitating role [283,284], Ca^{2+} alone is sufficient to induce MPT, probably via its double properties to bind to PTP regulatory sites [6,203,265] and, in addition, to stimulate the generation of oxidants by mitochondria [263,284–286]. Evidence has also been provided that other inducers such as phosphate [258], uncouplers [280], fatty acids [282] and statins [197,287,288] stimulate mitochondrial oxidant generation [266].

Indeed, various mitochondrial proteins claimed to participate in PTP formation are amenable to cysteine oxidation by mitochondrially-generated oxidants [289]. In line with these findings, redox signals mediated through cysteine oxidation via sulfenylation, sulfinylation, S-glutathionylation and S-nitrosylation regulate mitochondrial functions such as oxidant production, MPT, apoptosis and mitochondrial fission and fusion [289].

With respect to the mechanisms by which matrix Ca^{2+} stimulates oxidant generation, Ca^{2+} binding to sub-mitochondrial particles, probably to cardiolipin, was shown to cause important alterations in membrane lipid organization [285]. These alterations were characterized by increased lipid packing and lipid lateral phase separation associated with free radical production at the electron transport chain. This was consistent with the increased production of H_2O_2 when matrix Ca^{2+} levels are elevated leading to oxidative alterations of the mitochondrial inner membrane [285] (see Fig. 2).

4.5. The role of nicotinamide nucleotide transhydrogenase in Ca^{2+}-dependent MPT

The continuous re-reduction of $NADP^+$ is of critical importance for the maintenance of mitochondrial redox balance since NADPH donates electrons to enzymatic systems involved in the detoxification of peroxides and reduction of thiols in mitochondria [290,291]. The two main mitochondrial sources of NADPH are the NNT and NADP- dependent isocitrate dehydrogenase (IDH2) [149,291,292]. While IDH2 is a soluble enzyme that promotes the oxidation of a carbon substrate in the Krebs cycle, the NNT is located in the IMM and catalyzes the reversible transfer of hydride (H^-) between NAD and NADP, reducing one at the expense of the oxidation of the other [292,293]. H^- transfer from NADH to $NADP^+$ via NNT is coupled to H^+ translocation through the IMM (from the IMS to the mitochondrial matrix). Thus, the existing ΔP across the IMM displaces NNT activity towards $NADP^+$ reduction and NADH oxidation, making its forward activity prevail under most metabolic conditions [149,292,294]. Under resting conditions (in the absence of ATP formation) characterized by high ΔP and elevated NAD redox potential, thermodynamic equations predict that NNT activity results in a NADPH:$NADP^+$ ratio that exceeds five hundred-fold the ratio of NADH:NAD^+ in the mitochondrial matrix [294]. Accordingly, it has been shown that decreases in NAD redox potential and in ΔP, such as those prevailing under conditions of increased ADP phosphorylation rates or decreased availability of respiratory substrates, may limit the forward NNT activity or even promote the reversal of its activity (in this case, NNT oxidizes mitochondrial NADPH and pumps H^+ out of the matrix) [149,178,295].

Our group has recently demonstrated that the ability of NNT to supply NADPH for peroxide removal in isolated liver mitochondria is highly dependent on energy state and on substrate availability [149]. Interestingly, when the thermodynamics disfavor forward NNT activity during ADP phosphorylation, other concurrent sources increase their own contributions toward NADPH supply (see Fig. 4). In this manner, the mitochondrial capacity to remove peroxide is totally or partially preserved as compared to resting conditions, depending on exogenous substrates fed to the Krebs cycle [149]. ADP-stimulated respiration will result in higher oxidation rates of carbon substrates, thus increasing IDH2 flux and the provision of NADPH by this Krebs cycle enzyme. Mitochondrial glutamate dehydrogenase may also reduce $NADP^+$, and this activity is strongly dependent on ADP, which makes this enzyme a source of NADPH that sustains high rates of NADPH-dependent peroxide removal in liver mitochondria during ADP-stimulated respiratory states in the presence of glutamate as a substrate [149]. Another mitochondrial source of NADPH are NADP-linked malic enzymes, whose specific rates in mouse liver ($nmol_{NADPH}/mg_{protein}/min$) are similar to that of NADP-linked glutamate dehydrogenase [149]. Nonetheless, more research is needed to uncover the relevance of NADP-linked malic enzymes for NADPH supply under different metabolic conditions. It is noteworthy that recent data strengthened the view that cytosolic NADP redox potential is not connected to that of the mitochondrial matrix, and vice-versa [296].

Given the NNT properties highlighted above, the mitochondrial energy state could be presumed to affect the redox state of NADP and several related processes such as scavenging of peroxides and Ca^{2+}-dependent MPT pore opening. We will present bellow important findings that uncovered the roles of NNT in the modulation of MPT pore opening. Vercesi manipulated NAD redox state and ΔP in isolated liver mitochondria in order to oxidize NADPH via the displacement of the NNT reaction [178]. The oxidation of NADPH that occurs following NADH oxidation and concomitant dissipation of ΔP resulted in mitochondrial Ca^{2+} release, which is currently interpreted as a consequence of MPT pore opening [178]. The exclusive oxidation of NADH in polarized mitochondria also stimulated Ca^{2+} release, but to a much lower extent in comparison to NADPH oxidation [178]. Despite knowing that NAD(P)H oxidation prompted mitochondrial Ca^{2+} release [40], these data [178] provided early evidence that the redox state of NADP, rather than that of NAD, plays a role in Ca^{2+}-dependent MPT pore opening. Another implication of the reported findings was the involvement of NNT in modulating MPT pore opening under specific mitochondrial energy conditions. In addition to being considered (already at that time) a central source of NADPH required for redox balance [294,297], the demonstrated reverse activity of NNT fitted well with the explanation of why fatty acid- or protonophore-induced uncoupling elicited MPT pore opening in Ca^{2+}-loaded mitochondria [282], an energetic condition in which ΔP drops and NADH is oxidized by increased respiratory chain complex I activity. The biological relevance of reverse NNT activity as a cause of oxidative stress has been recently documented in an *in vivo* condition encompassing elevated energy demand [295]. Nickel et al. [295] used mice lacking NNT activity (due to a mutated $Nnt^{C57BL/6J}$ allele) to demonstrate that the reverse activity of this enzyme aggravates redox imbalance and myocardial necrosis in a heart failure model induced by pressure overload. These authors also performed pharmacological interventions targeting mitochondria and the results obtained may suggest that the myocardial injury induced by the reversal of the NNT activity involves MPT pore opening [295].

Importantly, an *Nnt* mutation is present in mice used in the above mentioned study [295]. This mutation arose spontaneously more than three decades ago in the widely used C57BL/6J mice substrain colony maintained in The Jackson Laboratory [298,299]. Thus, this mutated allele has been named $Nnt^{C57BL/6J}$ in the Mouse Genomic Informatics database. Nonetheless, the discovery of this *Nnt* mutation in the most used mouse substrain only occurred in 2005 [300,301], and relatively little attention has been paid to potential scientific issues [299,302]. Although the unaware use of mice that carry mutated *Nnt* may cause research bias [299,303–306], mice lacking NNT due to this mutation have recently been employed in studies that aimed at uncover NNT functions in different experimental setups [295,303,307–309].

We were the first to employ *Nnt* mutant mice in order to describe the consequences of absent NNT activity for specific mitochondrial redox processes and Ca^{2+}-induced MPT pore opening in isolated liver mitochondria [307]. Indeed, we showed that lack of NNT facilitates Ca^{2+}-induced MPT pore opening under experimental conditions that are associated with spontaneous NADPH oxidation over time [307]. Conversely, there was no influence of NNT activity on Ca^{2+}-induced MPT pore opening when the supplied respiratory substrates were able to sustain NADP in its reduced state, which may have occurred mainly via the activity of IDH2 [307]. Therefore, forward NNT activity seems to play a critical role in providing mitochondrial NADPH and protection against Ca^{2+}-induced MPT pore opening in isolated mitochondria when the activities of concurrent NADPH sources are not favored by the available substrates [307].

After establishing a suitable mouse model for *in vivo* interventions [149], we also attempted to investigate whether NNT would play a role in the pathogenesis of non-alcoholic fatty liver disease induced by a high fatty diet [303]. Mitochondrial redox imbalance has generally been assigned as an important factor involved in the etiology of non-alcoholic fatty liver disease (NAFLD) [310]. Indeed, our study supported this assertion, since the *Nnt* mutation aggravated mitochondrial redox imbalance and caused NAFLD progression from simple steatosis to steatohepatitis in mice fed a high fatty diet [303]. Interestingly, the *Nnt* genotype greatly modified the effects of 20 weeks of high fatty diet on Ca^{2+}-induced MPT pore opening, as evaluated in isolated mitochondria from livers *in vitro*. While the high fatty diet surprisingly decreased the susceptibility to Ca^{2+}-induced MPT pore as compared to chow-fed wild type mice, the high fatty diet intervention in *Nnt*-mutant mice led to an increased susceptibility to Ca^{2+}-induced MPT [303]. Overall, these findings evidenced the critical roles of NNT in the attenuation of mitochondrial redox imbalance, MPT pore opening propensity and non-alcoholic fatty liver disease development in response to high fatty diet in experimental mice [303].

4.6. Modulation of Ca^{2+}-dependent MPT pore opening by reactive nitrogen species (RNS)

It must be emphasized that that $^{\bullet}NO$ became established as a relevant physiological molecule involved in a variety of cellular processes only in the late 1980s [151]. From then on, the research field related to $^{\bullet}NO$ evolved rapidly and the interaction between $^{\bullet}NO$ or $ONOO^-$ with mitochondria started to be investigated in the 1990s [162,165,166,311]. A seminal report from Radi et al. [158], published early in 1994, showed that exogenous $ONOO^-$ inhibited the activity of respiratory complexes I and II in isolated mitochondria from heart, leading to decreases in the respiratory rates supported by glutamate/malate or succinate. Within this same year, others demonstrated that a main effect of $^{\bullet}NO$ on mitochondria was a partial, reversible, and competitive inhibition of complex IV [162], later proved to be of physiological relevance [312,313].

Other effects of $^{\bullet}NO$ on isolated mitochondria observed in early studies included Ca^{2+} release or failure to take up Ca^{2+}, which were generally associated with inhibition of respiratory chain activity and mitochondrial inner membrane depolarization promoted by elevated amounts of this radical [166,314]. These effects, however, are energy-associated and should not be considered primary. Because Ca^{2+} release from Ca^{2+}-loaded mitochondria following their depolarization may occur via different routes, *e.g.* secondarily to MPT pore opening, membrane damage or/and by the reversal of the MCU channel [178,314], proper experimental designs were needed in order to discriminate the different effects of $^{\bullet}NO$ on mitochondrial bioenergetics, Ca^{2+} handling and membrane integrity.

Considering the involvement of RNS in MPT, important data were also published in 1994. By showing that high levels of exogenous $^{\bullet}NO$ promoted CsA-sensitive mitochondrial depolarization, Ca^{2+} release and death in isolated hepatocytes, Richter et al. [311] provided early experimental evidence suggesting that RNS may indeed play a role in the modulation of Ca^{2+}-induced MPT pore opening. Notably, CsA was already known as an inhibitor of Ca^{2+}-induced MPT pore opening [184]. Also in 1994, Packer and Murphy [315] published a study where they exposed isolated rat liver mitochondria directly to the more reactive $ONOO^-$, hypothesizing that mitochondria may be a target of $ONOO^-$ (and derived reactive species) generated from the combination of $^{\bullet}NO$ with $O_2^{\bullet-}$ under conditions such as inflammation or ischemia/reperfusion. Indeed, their results indicated that a bolus load of $ONOO^-$ promoted Ca^{2+} efflux from mitochondria in a CsA-sensitive manner, thus implying that MPT pore opening can be facilitated in the presence of $ONOO^-$ [315]. As reviewed above, it is important to remember that, at this time, seminal studies had already paved the road toward the concept of Ca^{2+}-induced MPT as a redox-sensitive process – research findings linked oxidants to mitochondrial membrane protein thiol oxidation and MPT pore opening upon Ca^{2+} exposure [40,178,216]. Within this context, some researchers went on to further investigate how the two most known RNS, $^{\bullet}NO$ and $ONOO^-$, affected Ca^{2+}-induced MPT pore opening.

Using an experimental setup in which isolated mitochondria were loaded with Ca^{2+} and co-incubated with exogenous sources of $^{\bullet}NO$ and $O_2^{\bullet-}$ to fuel the continuous and steady formation of $ONOO^-$, the combination of those two radicals elicited Ca^{2+} efflux as a consequence of MPT pore opening [316]. Although the data were not shown, the authors of this study stated that neither $^{\bullet}NO$ nor $O_2^{\bullet-}$ alone caused Ca^{2+} efflux [316]. In subsequent work, $ONOO^-$-induced mitochondrial glutathione and NAD(P)H oxidation were shown to occur irrespective of Ca^{2+}-dependent MPT pore opening [317], indicating that these redox changes are not the consequence of MPT pore opening. In comparison to mitochondrial glutathione oxidation and disulfide bond formation induced by a common exogenous peroxide (*i.e. t*-BOOH), $ONOO^-$ promoted non-recoverable glutathione oxidation and increased levels of protein-glutathione mixed disulfides [317]. As discussed in this review, direct oxidation of the mitochondrial thiol pool or of NADPH may explain why $ONOO^-$ stimulates Ca^{2+}-dependent MPT pore opening. In fact, the role of thiol oxidation under these conditions was highlighted by the fact that a thiol reducing agent (i.e. DTT) reversed the effects of $ONOO^-$ [317]. Ca^{2+}-dependent MPT pore opening may not be the only process occurring when mitochondria are exposed to $ONOO^-$: other dysfunctions such as oxidation and inhibition of mitochondrial enzymes and of the respiratory chain, as well as Ca^{2+}-independent permeabilization of inner membrane, may also occur [165,318,319]. We showed that the latter effect of $ONOO^-$ resulted from membrane protein oxidation and aggregation due to membrane protein thiol cross-linking and lipid peroxidation [318].

The cytotoxic role of $^{\bullet}NO$ was reinforced when Kroemer's group used different $^{\bullet}NO$ donors (e.g., S-nitrosoglutathione) and demonstrated that this radical promoted apoptosis mediated by MPT pore opening in intact thymocytes [320], thus corroborating the early findings from Richter et al. [311]. $^{\bullet}NO$ is the primary RNS generally linked to physiological functions, but also a precursor of $ONOO^-$ in the presence of $O_2^{\bullet-}$. Thus, despite these early literature findings, the effects of $^{\bullet}NO$ *per se* and of different $^{\bullet}NO$ levels on Ca^{2+}-dependent MPT pore opening or on cell death were still uncertain. In this context, questions remained as to whether $^{\bullet}NO$ behaves only as an inducer of Ca^{2+}-dependent MPT pore opening.

Brookes et al. [321] attempted to describe the effects of varying concentrations of $^{\bullet}NO$ on Ca^{2+}-dependent MPT. These authors reported that lower levels of $^{\bullet}NO$ protected against MPT pore opening, while high doses stimulated it. Because the observed protection was associated with energy-related attenuation of mitochondrial Ca^{2+} uptake (i.e. secondary to respiratory inhibition), it remained unclear whether lower amounts of $^{\bullet}NO$ could also directly regulate the molecular processes leading to MPT pore opening. Important clues that $^{\bullet}NO$ could provide mitochondrial and cellular protection against Ca^{2+} overload came from later studies investigating thiol modifications by $^{\bullet}NO$ and the formation of S-nitrosylated proteins in mitochondria and heart tissue [322–327]. Targeted thiols in proteins cysteine residues and reduced gluthatione can establish a covalent bond with $^{\bullet}NO$, forming S-nitrosothiols (R-SNO), the product of S-nitrosylation [167,325]. S-nitrosylation is a post-translational modification of some proteins that may regulate their activity [323]. Ohtani et al. [328] used various concentrations of NONOate as an $^{\bullet}NO$ donor and demonstrated that lower levels of NONOate attenuated atractyloside-induced Ca^{2+}-dependent MPT pore opening via mechanisms related to S-nitrosylation and decreased thiol oxidation/disulfide bond formation. Importantly, these authors also showed that this effect was not secondary to changes in mitochondrial membrane potential and that the facilitation of MPT pore opening in the presence of high amounts of NONOate was abolished by a chemical scavenger of $O_2^{\bullet-}$ and $ONOO^-$ [328]. Therefore, it seemed clear that $^{\bullet}NO$ could play dual roles in MPT pore opening: i) facilitation may occur when high levels result in formation of its derivative and more oxidant RNS $ONOO^-$; ii) protection may happen when S-nitrosylation of mitochondrial proteins avoids the oxidation of putative critical thiols. The details about the molecular mechanisms by which S-nitrosylation of mitochondrial proteins protects against Ca^{2+}-induced MPT pore opening were elegantly described by Nguyen et al. [329]. Knowing that cyclophilin D is a critical regulator of MPT pore opening and that this protein is S-nitrosylated at cysteine 203 upon exposure to S-nitrosoglutathione [325], Nguyen et al. [329] performed mutation studies and showed that cysteine 203 of cyclophilin D was a target of S-nitrosylation required for the attenuation of H_2O_2-triggered MPT pore opening in fibroblast cells following their treatment with nitrosoglutathione. This and other studies seem to indicate that oxidation of protein thiols, formation of disulfide bonds and protein cross-linking may be lessened by $^{\bullet}NO$-mediated S-nitrosylation [322,328,329]. In this regard, there is strong experimental evidence that cardioprotection following interventions such as administration of $^{\bullet}NO$ precursors or ischemic pre-conditioning is associated with increased S-nitrosylation of proteins and hindered thiol oxidation

[156,324,326].

5. MPT and metabolic disturbances

We will now discuss mitochondrial dysfunctions that lead to MPT in the context of dyslipidemias, diabetes, obesity and related conditions. The set of findings presented here strongly suggests that prevention of MPT may be a suitable target to therapeutically impact upon these metabolic diseases.

5.1. Hypercholesterolemia and MPT: relevance to atherogenesis

Familial hypercholesterolemia is a genetic disorder inherited in an autosomal dominant pattern and characterized by high plasma cholesterol levels, specifically in the low-density lipoprotein (LDL) fraction, and early cardiovascular disease due to atherosclerosis. Familial hypercholesterolemia is caused by mutations in the LDL receptor gene (*Ldlr*) that encodes a receptor that normally removes LDL particles from the circulation. Subjects who have one mutated allele (heterozygous) of the *Ldlr* gene may have premature cardiovascular disease at the age of 30–40. Two mutated alleles (homozygous) may result in severe cardiovascular disease and death during childhood. Heterozygous familial hypercholesterolemia is very common, occurring in 1:200–1:500 people in most countries, whereas homozygous is much rarer, occurring in 1 in a million births [330].

The mechanism in which excess circulating LDL-cholesterol relates to atherosclerosis is explained by the "oxidative modification hypothesis" that proposes that LDL is a major target of oxidation in the arterial subendothelial space and is involved in both initiation and progression of atherosclerosis [331,332]. Oxidized LDL induces endothelium activation and recruitment of circulating monocytes, which differentiate into macrophages that continuously take up oxidized LDL turning into cholesterol-engorged foam cells, the hallmark of atherosclerosis [333]. The process evolves with unresolved inflammatory features and foam cells die, forming the necrotic core of advanced lesions. In this condition of hypercholesterolemia, there is strong support for the occurrence of vascular wall oxidative stress that triggers LDL oxidation; however, the multiple possible mechanisms that drive *in vivo* LDL oxidation have not been completely characterized yet [334]. We have previously investigated the possible contribution of mitochondria to cellular oxidative stress in a familial hypercholesterolemia model, the atherosclerosis-prone *Ldlr* knockout mice (LDLr-/-). Mitochondria from several tissues in these mice generate more oxidants than controls and are more susceptible to Ca^{2+}-induced MPT [335]. These findings reveal that mitochondrial redox imbalance could indeed be involved in two key events of atherosclerosis: i) as a source of oxidants that oxidize LDL and ii) as the mitochondrial pathway for cell death [336]. We have also confirmed in naïve hypercholesterolemic subjects that oxidants derived from peripheral blood monocytes, preferentially from mitochondria, were increased along with oxidized LDL plasma levels [337]. Mitochondrial oxidative stress and enhanced MPT response was also shown in the porcine myocardium of hypercholesterolemic pigs [338]. We demonstrated that mitochondrial oxidative stress in LDLr-/- mice is associated with the depletion of mitochondrial NADP-linked substrates which leads to insufficient amounts of reducing equivalents (NADPH) to reconstitute the H_2O_2 scavenging function of the glutathione and thioredoxin reductase/peroxidase system [339]. Indeed, mitochondrial NADPH deficiency, oxidant accumulation and MPT could be partially reversed by treatment with isocitrate, catalase [339] and with the natural antioxidants Mangiferin and Vimang [340,341]. The NADPH deficit in LDL receptor-defective cells can be in part explained by the augmented cholesterol synthesis in these cells [335], a pathway that consumes large amounts of NADPH (24:1 molar ratio). Using several *in vivo* treatments of LDLr-/- mice in an attempt to spare mitochondrial NADPH content (citrate, pravastatin, citrate + pravastatin), we were able to modulate the mitochondrial oxidant production rates, which correlated with the severity of atherosclerosis [342]. The positive correlation between mitochondrial oxidant production rates and the size of aortic atherosclerotic lesions in this model was also verified in the context of aging [343]. In agreement, increased MPT response to Ca^{2+} in hypercholesterolemic pigs was associated with decreased levels of reduced glutathione (GSH) and of antioxidant enzymes activities (MnSOD, thioredoxin and peroxiredoxin), as well as with decreased expression of putative MPT pore components mitochondrial phosphate carrier and cyclophilin-D [338].

In an attempt to increase mitochondrial antioxidant capacity, we treated hypercholesterolemic LDLr-/- mice with statins to inhibit cholesterol synthesis and prevent NADPH depletion. Statins are widely used hypocholesterolemic medicines that are effective in reducing cardiovascular disease mortality. Unexpectedly, lovastatin treatment increased the susceptibility of liver and muscle mitochondria to Ca^{2+}-induced MPT. In isolated mitochondria, statin effects were shown to be class and dose-dependent and were associated with the oxidation of protein thiol groups [288], a key event in the formation of the PTP [216]. These data indicated that statins might act directly on mitochondria, leading to MPT, that in turn may ensue cell death. In fact, we observed that simvastatin induces prostate cancer PC3 cell death, either by apoptosis (low dose) or necrosis (high doses). The partial prevention of necrosis by MPT inhibitors indicated PTP opening in the mechanism of cancer cell death [344]. These effects of simvastatin on PC3 cells were later shown to be sensitive to piracetam (a membrane stabilizer) and L-carnitine (an antioxidant) [345]. Others also have shown statin-induced tumor cell death [346]. Although statins are considered adjuvants in the treatment of cancer, more studies are needed to better clarify this matter [347].

It is noteworthy that 0.5–10% of the statin-treated patients present dose- and class-dependent adverse effects, mainly related to the skeletal muscle. Our group showed that addition of simvastatin (1 μM) to rat soleus muscle biopsies decreased the content of CoQ10 and inhibited mitochondrial respiration within one hour [287]. Interestingly, L-carnitine or CoQ10 co-incubation with simvastatin, both of which can act as free radical scavengers, protected muscle mitochondria against respiratory inhibition [287]. Data from this and other studies [348] suggested that the respiratory dysfunction induced by simvastatin was mainly promoted by the attack of mitochondrially-generated superoxide radicals to complexes I and II, likely at their 4Fe-S clusters. Afterwards, we went back to the more relevant *in vivo* context of the familial hypercholesterolemia model, the LDLr-/- mice. We studied chronic treatment (three months) with moderate therapeutic doses of a less toxic and hydrophilic statin, pravastatin [197]. We observed that respiratory rates were inhibited in the presence of free Ca^{2+} in plantaris muscle (but not in soleus) from treated LDLr-/- mice. Such a decrease in respiratory rates was abolished in the presence of Ca^{2+} chelator EGTA, mitochondrial Ca^{2+} uptake inhibitor ruthenium red, and MPT inhibitor cyclosporin A (CsA). These findings indicate that *in vivo* pravastatin inhibited mitochondrial respiration and induced Ca^{2+}-dependent PTP opening in plantaris muscle. Along with the respiratory dysfunction and MPT, pravastatin treatment also caused lipid oxidative damage and increased catalase activity. All these pravastatin effects are counteracted by co-treatment with the antioxidants CoQ10 and creatine. These findings suggest that oxidative stress provoked by pravastatin signals for the upregulation of cellular antioxidant systems (e.g. catalase). When such adaptive responses are not sufficient to counteract oxidative imbalance, PTP opens and cell death may occur [197]. In summary, statin actions on mitochondria are both direct and indirect, as follows: i) in mitochondrial preparations, statins induced oxidation of protein thiol groups (50% decrease of free SH-) that was prevented by the reducing agent DTT (dithiothreitol) or by the Ca^{2+} chelator EGTA [288]; ii) in muscle biopsies, statin suppression of the mevalonate pathway induced mitochondrial dysfunctions (inhibition of respiration, increase of H_2O_2 accumulation, decrease of CoQ10 content) that were reversed by either mevalonate or CoQ10 replacement [287]; and iii) in chronic

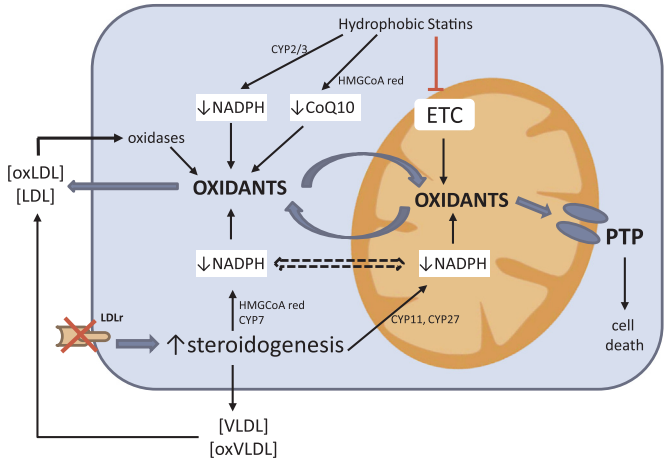

Fig. 6. Mechanisms by which hypercholesterolemia due to LDLr deficiency and statin treatment increase oxidant production and decrease antioxidant capacity, increasing PTP formation. LDLr deficiency induces a compensatory increase in cholesterol synthesis and metabolism. Thus, several pathways that consume reducing power (NADPH) are upregulated such as mevalonate (HMGCoA reductase), bile acid (CYP7) and cholesterol oxidation (CYP11/27). Decreased mitochondrial and cell NADPH impairs mitochondrial antioxidant enzymatic systems and favors oxidant accumulation and PTP formation. Liver increased steroidogenesis and lipogenesis results in enhanced VLDL secretion rates, which include oxidized (ox) components. Augmented VLDL contributes to further increases in extracellular LDL and oxLDL. Additionally, permeable mitochondrial and cell oxidants may directly attack LDL, generating oxLDL, which in turn are taken up by cells and activate extra mitochondrial oxygenases (ex.: NADPH oxidase), favoring oxidant production. Statin treatment reduces steroidogenesis but also reduces the synthesis of the antioxidant CoQ10 (mevalonate pathway intermediate). Statins may also interact directly with mitochondrial respiratory complexes inhibiting respiration and increasing oxidant accumulation. Hydrophobic statins are metabolized in the liver by the cytochrome P450 enzyme system (CYP2/3), which are NADPH-dependent monooxygenases.

in vivo treatment, statin elicited both pro- and antioxidant responses such as oxidized lipids and catalase upregulation, respectively, in skeletal muscle [197]. Additionally, in liver, hydrophobic statins are metabolized by the cytochrome P450 enzyme system (CYP2/3), which are NADPH dependent monooxygenases, thus favoring the depletion of this important reducing power.

The major findings and evidences described in this Section are summarized in Fig. 6.

5.2. Diabetes increases MPT

Diabetes mellitus is a very common metabolic disease [349]. The complications associated with diabetes lead to low quality of life and high incidence of micro- and macro-cardiovascular diseases. Prolonged hyperglycemia alone, or in combination with hyperlipidemia, seems to be the trigger of the cellular toxic events that occur in several tissues besides the vessels, such as the myocardium, liver, muscle and pancreatic beta cells of diabetic patients. Mitochondrial respiratory rates, susceptibility to oxidative stress and to MPT are altered in several hyperglycemic experimental models of type 1 and type 2 diabetes, such as high fat diet- or drug-induced, as well as in hereditary diabetes (GK and Fa/fa rats and NOD and db/db mice).

In Type 1 diabetes (T1D), the autoimmune destruction of pancreatic beta cells is associated with cellular oxidative stress and mitochondrial cell death pathway activation [350]. We have previously studied mitochondrial function of spontaneous non-obese diabetic (NOD) mice [351]. The establishment of T1D in NOD mice is similar to what occurs in humans, beginning with inflammatory cell infiltration into pancreatic islets followed by activation and release of inflammatory cytokines and advancement of pancreatic beta cell death along with the loss of glycemic homeostasis [352]. Thus, we studied NOD mice at three stages: non-diabetic (age 4–6 weeks, glycemia < 100 mg/dl), pre-diabetic (age 7–10 weeks, glycemia 100–150 mg/dl), and diabetic (age 14–25 weeks, glycemia > 250 mg/dl) as compared with age-matched Balb/c control mice. Global cell oxidant levels, but not mitochondrial oxidant production, was significantly increased in NOD lymphomononuclear and bone marrow stem cells in all three stages. In addition, pancreatic islets from non-diabetic NOD mice (the only stage when it is possible to obtain apparently intact islets) already showed a markedly elevated oxidant production. Isolated NOD liver mitochondria were more susceptible to Ca^{2+}-induced MPT at all three stages of spontaneous diabetes development. These results suggest that cellular oxidative stress precedes diabetes and may be the cause of mitochondrial dysfunction that is involved in beta cell death [351].

Mutations of the pancreatic duodenal homeobox gene-1, *Pdx1*, cause heritable diabetes in humans and mice. Suppression of *Pdx1* increased mouse insulinoma MIN6 beta-cell death associated with dissipation of the mitochondrial inner membrane electrochemical gradient. Preventing mitochondrial PTP opening with CsA restored mitochondrial membrane potentials and rescued cell viability [353]. In addition, genetic ablation of the gene encoding cyclophilin D (a PTP pore opening facilitator) in *Pdx-1* deficient mice restored beta-cell mass, inhibited apoptosis and necrosis and normalized diet-induced disturbances in glucose homeostasis [353].

Type 2 diabetes (T2D) is generally linked to prolonged exposure to unbalanced diets, hyperlipidemias and obesity. Low-grade inflammation seems to be the common denominator under these conditions, leading to disruption of insulin signaling in the insulin target tissues, resulting in insulin resistance. This state overloads pancreatic beta cells demanding high insulin production rates to cope with the hyperglycemia caused by the peripheral resistance to hormone action. Thus, in T2D, hyperinsulinemia coexists with hyperglycemia up to the stage where pancreas exhaustion occurs. Insulin resistance is associated with mitochondrial dysfunction [354], but the specific mechanisms are still not completely elucidated and may differ according to the insulin target tissue and the type of insulin resistance inducer. The opening of mitochondrial PTP has been shown to play a critical role in defective insulin-mediated glucose uptake in T2D. *In vitro* models of insulin resistance have reinforced this idea. In several models of insulin resistance in cultured skeletal muscle cells (antimycin A, C2-ceramide, ferutinin, and palmitate), the pharmacological inhibition of PTP opening with CsA was sufficient to prevent insulin resistance at the level of insulin-stimulated GLUT4 translocation to the plasma membrane [355].

In vivo, there are several examples of diet-induced insulin resistance and/or hyperglycemia promoting MPT. Prolonged exposure to high-fat/high-sucrose diet results in increased oxidant production associated with mitochondrial morphological and functional alterations in the skeletal muscle of hyperglycemic mice. Decreased phosphorylating and resting respiration rates as well as mitochondrial swelling (an indicator of MPT) were observed in the muscles of these mice. Normalization of glycemia or antioxidant treatment decreased muscle oxidant production and restored mitochondrial integrity [356]. The same mitochondrial alterations, including swelling and disruption, were observed in the skeletal muscle of streptozotocin-treated mice, a known model of hyperglycemia-induced oxidative stress [356].

In rats fed a high fat/high fructose diet as an insulin resistance inducer and submitted to an acute stress, there was a marked decrease in the amount of Ca^{2+} required to open the PTP in brain mitochondria, suggesting an adverse effect of insulin resistance on brain mitochondrial survival [357].

Db/db mice lacking the leptin receptor are murine models of genetic spontaneous T2D that develop hyperphagic obesity in addition to diabetes. Early studies with this model showed that their cardiac muscle cells contain large numbers of lipid droplets and exhibit degeneration of

mitochondria [358]. Lanthanum-treated myocardial tissue clearly demonstrates an abnormal permeability of sarcolemmal and mitochondrial membranes of cardiomyocytes from these diabetic mice [359]. Together with these ultrastructural alterations, defects in fatty acid oxidation resulting in accumulation of beta-hydroxy fatty acid intermediates in the myocardium and defects in NAD^+-linked substrate utilization by isolated mitochondria were also observed [359]. As mitochondrial oxidant production has been implicated in the pathogenesis of diabetic cardiomyopathy [360,361], the therapeutic effects of the mitochondria-targeted antioxidant mito-TEMPO have been investigated in T1D (streptozotocin treatment) and T2D (db/db mice) diabetes models. Indeed, one month of daily injections of mito-TEMPO inhibited mitochondrial oxidant levels, prevented intracellular oxidative stress, decreased apoptosis and reduced myocardial hypertrophy in these diabetic hearts [362].

Zucker Fa/fa rats (Fa/fa) present T2D, obesity and cardiomyopathy. Riojas-Hernández et al [363] showed a prolonged systolic Ca^{2+} transient and markedly increased mitochondrial H_2O_2 production in Fa/fa rat myocardium. Mitochondrial PTP opening in isolated heart mitochondria from Fa/fa rats was more sensitive to Ca^{2+} than mitochondria from lean control rats and correlated with increased thiol group exposure. Fa/fa mitochondria have decreased oxidative phosphorylation capacity, explaining the decreased ATP content in their myocytes. The results thus suggest that oxidative stress and Ca^{2+} dysregulation increased MPT sensitivity leading to mitochondrial dysfunction and apoptosis [363]. In the Goto-Kakizaki (GK) rats, a non-obese Wistar substrain that develops T2D early in life, MPT has been documented in mitochondria from brain, kidney [364,365] and heart [366,367].

One of the best opportunities to explore the role of MPT as a determinant of metabolically-disturbed phenotypes is the knockout (KO) of CypD, a critical regulator of the PTP [368]. As expected, muscle mitochondria from CypD KO were shown to be resistant to $Ca2^+$ induced MPT, as evidenced by resistance to fatty diet-induced swelling and improved mitochondrial calcium retention capacity compared to controls [355]. These CypD KO mice did not develop glucose intolerance, had improved skeletal muscle glucose uptake following high-fat feeding [355,369] and were resistant to diet induced obesity [370]. However, conflicting with these findings, two other studies have found that mice lacking CypD displayed an age-related development of obesity [371] and hepatic insulin resistance [372]. Therefore, the scenario is more complex than had been anticipated and probably involves tissue-specific [373] and/or MPT-independent roles of CypD.

Advanced glycation end-products (AGEs) are thought to accumulate with aging. Under conditions such as hyperglycemia and hyperlipidemias, AGEs can be increased far beyond normal levels. They are toxic to nearly every type of cell in the body and are also believed to play a causative role in diabetes mellitus complications. AGEs elicit oxidative stress and cause inflammatory reactions via interaction with a receptor for AGEs (RAGE) [374,375]. Hyperglycemia leads to the generation of high levels of glucose metabolites, such as methylglyoxal, which react with proteins generating AGEs. In HepG2 cells, methylglyoxal promoted the production of oxidants, depleted glutathione content and induced apoptosis. Methylglyoxal treatment also induced MPT and pretreatment with CsA partially inhibited cell death induced by methylglyoxal [376]. In osteoblastic MC3T3-E1 cells, methylglyoxal increased mitochondrial superoxide and cardiolipin peroxidation, increased MPT and decreased intracellular ATP. The antioxidant trolox and CsA prevented methylglyoxal-induced cytotoxicity [377]. In addition, exposure of primary renal cells to AGEs, transient overexpression of the receptor for AGEs (RAGE) and infusion of AGEs in healthy rodents each induced renal cytosolic oxidative stress leading to MPT. When these experiments were performed in hyperglycemic conditions *in vitro* or in diabetic rats, significant generation of mitochondrial superoxide at the level of complex I was also observed. These results suggest that the AGE-RAGE pathway induced-MPT plays a role in the development and progression of diabetic nephropathy [378].

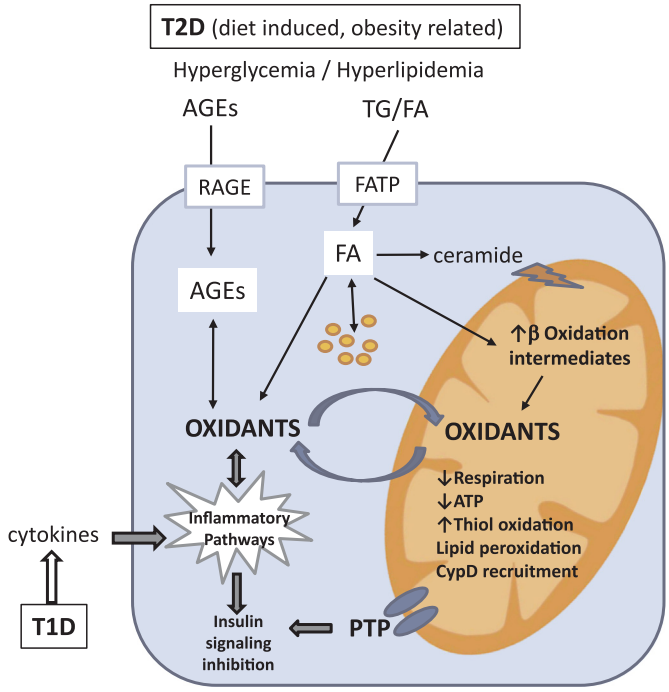

Fig. 7. Mechanisms in which type 1 (T2D) and type 2 (T2D) diabetes increase oxidant production, favoring PTP formation. Chronic hyperglycemia leads to nonenzymatic formation of advanced glycation end products (AGEs) that are internalized by cells through their receptors (RAGE) or formed intracellularly. AGEs induce and amplify oxidative stress. Hyperlipidemia, that generally is present in T2D, increases cellular facilitated transport of fatty acids (FATP, fatty acid transport proteins and others) that may exert several actions: 1) esterification and accumulation as lipid droplets, 2) increased ceramide synthesis, that is toxic to mitochondria, 3) form oxidants (enzymatic or nonenzymatic processes) and 4) enter mitochondrial β oxidation pathway. Overload of mitochondrial β oxidation without concomitant upregulation of electron transport results in increased oxidant production. In T1D, autoimmune attack of cytokines seems to be the primary insult generating cell oxidative stress. Mitochondrial dysfunctions in T1D and T2D include: reduced respiration rates and ATP production, increased protein thiol oxidation, lipid peroxidation, and PTP formation. Mitochondrial and extra mitochondrial oxidants induce and amplify inflammatory pathways that are responsible for insulin signaling inhibition. Inhibiting PTP rescues insulin sensitivity in several models (see text).

The major findings and evidences described in this Section are summarized in Fig. 7.

5.3. Fatty diets, obesity and MPT

Prolonged consumption of high fat diets or simply chronic overeating leads to an expansion of the body adipose tissue mass (obesity), insulin resistance and other co-morbidities. Lipid overload in tissues other than the adipose is believed to be the cause of many obesity co-morbidities (diabetes, cardiomyopathies, nonalcoholic fatty liver, etc). Tissue-specific lipotoxicity varies widely depending on diet composition, duration of feeding and the experimental model [379]. Mitochondrial dysfunctions have been investigated in several of these contexts. The mechanisms underlying mitochondrial dysfunctions are associated with intracellular overload of non-esterified fatty acids (NEFA). NEFA are classical mitochondrial uncouplers known for a long time [380,381]. They can act as protonophores and/or activating mitochondrial uncoupling proteins [380,382,383]. In excess, they are able to open the mitochondrial PTP through at least two mechanisms: i) they have the ability to interact directly with the adenine nucleotide translocator, and, ii) they induce mitochondrial oxidative stress [282,384]. Thus, when Ca^{2+}-loaded mitochondria are exposed to micromolar

concentrations of NEFA, the permeability of the inner membrane increases, resulting in matrix swelling, rupture of the outer membrane and release of intermembrane pro-apoptotic proteins [385].

The chronic consumption of obesogenic diets has been shown to cause heart mitochondrial uncoupling, oxidant production [386], alterations in Ca^{2+} oscillations [387], lower ATP/oxygen ratios associated with fatty acid oxidation [388] and synthesis of pro-apoptotic ceramides [389]. High-fat diet-triggered cardiac mitochondrial abnormalities (functional and structural), including susceptibility to MPT, have been consistently confirmed [361,390]. Whether these changes are a result of obesity-associated conditions such as insulin resistance (as discussed above) or related to the fatty diet more directly is difficult to distinguish. Littlejohns et al [391] have shown that a high fat diet (with low sucrose content) that induced enlargement of adipose depots and hyperlipidemia but did not cause insulin resistance indeed triggered changes in cardiac calcium handling and mitochondria that are likely to be responsible for the increased vulnerability to cardiac insults such as those promoted by ischemia-reperfusion. Compared to a normal diet, high-fat fed non-insulin resistant C57BL/6J mice presented higher $[Ca^{+2}]$i oscillations, higher catalase and lower SOD2 expression, smaller mitochondria and lower mitochondrial density in their myocardium. In addition, changes in the expression of putative components or regulators of the mitochondrial PTP were observed: increased inorganic phosphate carrier and decreased VDAC and hexokinase II, with no alterations in CypD and adenine nucleotide translocator. A role for MPT was demonstrated using CsA, that conferred cardioprotection against ischemia/reperfusion damages in the high fat-fed mice [391].

Liver mitochondria may be also damaged in high fat diet-induced non-alcoholic fatty liver, a common feature of obesity that evolves to nonalcoholic steatohepatitis. Enhanced mitochondrial fatty acid oxidation without concomitant up-regulation of the mitochondrial respiratory chain promotes oxidant overproduction. This event seems to play a significant role in the initiation of oxidative stress and subsequent development of nonalcoholic steatohepatitis [392].

We have previously employed a genetic mouse model that exhibits elevated plasma levels of triglycerides and NEFA and mild steatosis but no other metabolic confounding factors such as obesity and disturbed glucose homeostasis. Even under a balanced regular low fat diet, they already show increased liver mitochondrial susceptibility to Ca^{2+}-induced MPT [393]. Liver accumulation of NEFA was associated with this process, since *in vivo* treatment with the hypolipidemic drug ciprofibrate, which accelerates intracellular fatty acid beta-oxidation, inhibited MPT in the liver mitochondria of these hypertriglyceridemic mice [393]. When exposed to chronic high fat feeding, these mice showed exacerbation of obesity [394,395] and nonalcoholic steatohepatitis compared to controls [396], indicating that mitochondrial dysfunctions are early events that precede the appearance of the high fat diet-induced disease phenotypes.

Finally, it is important to emphasize that not only the content but also the quality of dietary fatty acids modifies mitochondrial function and integrity. For instance, a recent study investigated the trans-generational effect of dietary trans-fatty acids, a type of unsaturated fat that occurs in small amounts in nature, but is abundant in industrialized foods. Its intake has been associated with increased risk of coronary artery disease [397]. Compared to normolipidic diets, trans-unsaturated fatty acids and inter-esterified fat-containing diets administered to C57BL/6 mothers resulted in impaired liver mitochondrial respiration, increased H_2O_2 production and reduced mitochondrial Ca^{2+} retention capacity (MPT) in their offspring [398]. These results show evidence of an epigenetic impact of dietary fat on mitochondrial function.

5.4. Calorie restriction protects against MPT

Calorie restriction is a dietary intervention widely shown to prevent *ad libitum* feeding-induced obesity and increase lifespans, as well as prevent age-related diseases (reviewed by [399]). Recently, while studying mechanisms in which caloric restriction prevents excitotoxic neuronal cell death [400], we found that caloric restriction changes Ca^{2+} transport properties in brain mitochondria. Mitochondria isolated from the forebrains of mice maintained on a restricted diet exhibited resistance to MPT, an ability to accumulate larger amounts of Ca^{2+} and faster Ca^{2+} uptake rates. These results were correlated with a deacetylation of cyclophilin D, which inactivates this protein and prevents MPT. Based on these results and a prior study in skeletal muscle mitochondria suggesting high fat diets promote MPT [355], we hypothesized that dietary interventions could have a more widespread effect on mitochondrial Ca^{2+} homeostasis. Indeed, we found that caloric restriction also promoted a very significant increase in Ca^{2+} uptake capacity in liver mitochondria, with marked prevention of permeability transition [401]. Interestingly, in liver mitochondria the diet-induced changes in Ca^{2+} uptake could be completely eliminated by depleting and re-establishing intramitochondrial ATP levels, suggesting they are related to intramitochondrial adenine nucleotide levels, which both buffer Ca^{2+} and regulate permeability transition. Of note, caloric restriction also promotes cardiolipin redistribution among mitochondrial membranes [402], which may also play a key role in the prevention of permeability transition, given the role cardiolipin has in interacting with Ca^{2+} ions, as discussed above.

Overall, we believe that this newly found link between dietary interventions and mitochondrial Ca^{2+} makes metabolic sense, since both caloric intake and cellular Ca^{2+} homeostasis are pivotal metabolic regulators. We hope future studies will further uncover how changes in diet and mitochondrial Ca^{2+} are interrelated in both physiological and pathological metabolic regulation.

The major findings and evidences described in these two later Sections are summarized in Fig. 8.

In conclusion, stimulation of oxidant production seems to be the common denominator in metabolic disturbances that predispose to PTP opening. Upstream pathways that elevate oxidant production differ in each particular condition. In hypercholesterolemia and statin therapy, the depletion of reducing agents or antioxidant molecules (NADPH and CoQ10) are the key events. In diabetes, cytokine-induced inflammation and excess of oxidizable substrates (glucose and fatty acids) are the hallmarks. High fat diets, obesity and insulin resistance favor intracellular accumulation of free fatty acids that trigger a variety of toxic effects in both mitochondrial and extra-mitochondrial compartments. In addition, unbalanced diets are associated with perturbations of intracellular $[Ca^{2+}]$ that together with elevation of oxidants trigger PTP opening. Caloric restriction and some antioxidant treatments prevent or reverse the redox imbalance.

6. MPT and mitochondrial DNA

Mitochondria contain their own DNA. The human mitochondrial DNA (mtDNA) is 16.5 Kb long and encodes 2 rRNAs, 22 tRNAs and 13 polypeptides, which are subunits of 4 of the 5 oxidative phosphorylation (OXPHOS) complexes [403]. Mitochondrial DNA integrity is essential for mitochondrial function, as all 13 mtDNA-encoded polypeptides are required for proper function of respiratory complexes I, III and IV and the F_1F_O ATP synthase (reviewed in [404]). Mutations in the mtDNA or in nuclear-encoded OXPHOS subunits cause several human syndromes, collectively named OXPHOS diseases, which represent the largest group of inborn errors of metabolism [405], and have also been implicated in common diseases, particularly those associated with age [406].

The mtDNA is located in the mitochondrial matrix, as part of a nucleoproteic complex known as the mitochondrial nucleoid, likely containing 1–2 copies of the mtDNA and core protein components, including those required for mtDNA replication, repair and transcription, and peripheral proteins involved in several signaling pathways that control mitochondrial function [407]. Of relevance to this review,

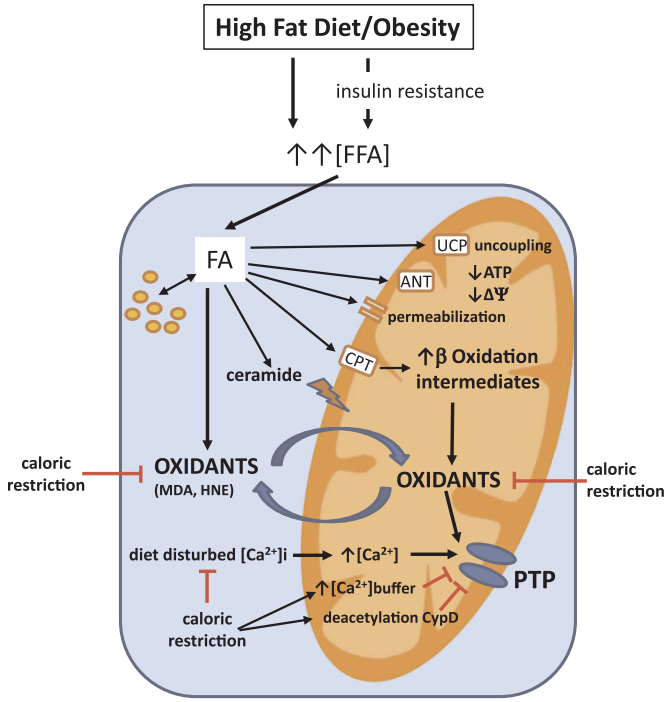

Fig. 8. Mechanisms in which high fat diet/obesity/insulin resistance increase oxidant production, favoring PTP formation. Increased exposure to high free fatty acids (FFA) concentrations results in uptake of fatty acids (FA) that may exert several actions: 1) esterification and accumulation as lipid droplets, 2) formation of oxidants (enzymatic or nonenzymatic processes) such as malondialdehydes (MDA) and 4-hydroxynonenal (HNE), 3) increased ceramide synthesis, that is toxic to mitochondria, 4) overload of mitochondrial β oxidation, producing intermediate excess and increasing oxidant production, 5) acting as protonophores, increasing membrane permeabilization, 6) interacting with adenine nucleotide translocator (ANT) and uncoupling proteins (UCP), decreasing the membrane potential and ATP production. Unbalanced high fat diets may also promote perturbations of intracellular [Ca^{2+}] handling by unknown reasons, which may contribute to increased mitochondrial Ca^{2+} and PTP formation. Caloric restriction may prevent PTP by 1) inhibition of oxidant production, 2) buffering diet induced [Ca^{2+}] perturbations, 3) promoting deacetylation of cyclophilin D (CypD) and 4) increasing intramitochondrial ATP levels.

mitochondrial nucleoids are tethered to the inner side of the IMM [408], in close proximity to sites of $O_2^{\bullet-}$ and H_2O_2 generation and to the PTP, making the mtDNA a prominent target for oxidation. In fact, several groups have demonstrated, in models such as flies, mice and humans, that mtDNA accumulates significantly more oxidized bases than nuclear DNA (reviewed in [409], although their relevance to mitochondrial function has been questioned recently (see [410]). It has been speculated that persistent MPT, and the ensuing redox imbalance, could lead to increased mtDNA damage, contributing toward mitochondrial bioenergetic dysfunction and cell death. Additionally, as mtDNA encodes essential OXPHOS components, mtDNA damage and mutations impact the electron transport chain, ATP production and mitochondrial redox state, suggesting a possible mechanistic link between mtDNA integrity and susceptibility to PTP opening and permeability transition.

Several lines of evidence support the idea that oxidants damage mtDNA, but few studies have directly addressed the role of MPT. We have addressed whether MPT causes mtDNA damage in isolated liver mitochondria exposed to the Fe^{2+}/citrate complex, which causes lipid peroxidation and membrane depolarization. This treatment induced intense mtDNA fragmentation, accumulation of 8-hydroxy-2-deoxyguanosine and 3′-phosphoglycolate termini, indicative of $^{\bullet}OH$-induced damage [411]. Exposure of human colon cancer cells to H_2O_2 also resulted in extensive mtDNA degradation, although the authors did not specifically demonstrate opening of the PTP under the experimental conditions used [412]. Recently, aflatoxin B1-induced mtDNA mutations in duckling livers were correlated with permeability transition, as indicated by mitochondrial swelling [413].

Other studies have linked permeability transition to nuclear DNA damage. Permeability transition was associated with extensive nuclear DNA fragmentation in a variety of experimental models including: i) angiotensin II-induced cardiomyocyte apoptosis [414]; ii) arsenite-induced DNA damage in cancer cell lines [415]; iii) oxidative DNA damage induced by oncogenic *ras* expression in normal human cells [416]; and iv) ionomycin-induced MPT in human spermatozoa [417]. While several of these studies correlated DNA damage induction to increased oxidant production, the observation that Ca^{2+}-induced mitochondrial membrane permeabilization causes Endonuclease G release from the mitochondrial intermembrane space [418] raises the possibility that, at least in part, DNA fragmentation is due to EndoG attack, as has been proposed for its role in apoptosis [419].

While still highly controversial, some authors have suggested that the c subunit of the F_1F_O ATP synthase is a component of the PTP. As two subunits of the ATP synthase, ATPase 6 and ATPase 8, are encoded in the mtDNA, mutations in these subunits could alter ATP synthase and impact on PTP opening. The mtDNA pathogenic mutation T8993G, found in high heteroplasmy in patients with neurogenic muscle weakness, ataxia, and retinitis pigmentosa (NARP), results in a Leu to Arg substitution in ATPase 6 subunit (subunit a), which is thought to, along subunit c, catalyze proton translocation and participate in the catalytic cycle. The mutation results in a structural defect in the F_1F_O ATP synthase and severe impairment of ATP synthesis [420]. Cybrids carrying 98% of T8993G mtDNA accumulate higher mitochondrial Ca^{2+} levels and showed increased oxidant production after hypoxia/reoxygenation [421]. However, the NARP cybrids responded to hypoxic preconditioning as control cells did, suggesting that the transient opening of the PTP involved in preconditioning is maintained in these mutants [422]. A recent study modeling two ATP6 mutations found in human cancers (ATP6-P136S and ATP6-K64E, found in prostate and thyroid cancer, respectively) in yeast showed that both mutations increased sensitivity of yeast cells to oxidative stress and to high calcium concentrations, and affected the activation of yeast permeability transition pore [423]. As for ATP8, while some variants have been associated with mitochondrial dysfunction and diseases (see www.mitomap.org/foswiki/bin/view/MITOMAP/MutationsCodingControl), the only confirmed pathogenic mutation in this locus, T8528C, overlaps the ATP6 gene [424], and no data is available on MPT in cells carrying this mutation. Altogether, most available data support the idea that mutations in mtDNA-encoded F_1F_O ATP synthase subunits render mitochondria more susceptible to permeability transition; however, none of these papers provide mechanistic evidence linking this effect to a direct role of F_1F_O ATP synthase subunits in the PTP.

Other mtDNA mutations that affect OXPHOS function could also indirectly impact MPT via increased oxidant production. Cybrid cells harboring pathogenic mtDNA mutations that cause MELAS (Myoclonic Epilepsy with Lactic Acidosis and Stroke, $tRNA^{Leu}$), MERRF (Myoclonic Epilepsy with Ragged Red Fiber, $tRNA^{Lys}$) and LHON (Leber's Hereditary Optic Neuropathy, ND1) were more sensitive to H_2O_2 than their wild-type cybrid controls, in a Ca^{2+}-dependent manner. In these experiments, cell death was significantly prevented by CsA, suggesting that MPT was involved in the citotoxicity mechanism [425].

On the other hand, somatic mtDNA mutations do not seem to associate with increased susceptibility to MPT. Data from two transgenic mouse models with increased mtDNA mutagenesis due to expression of a proofreading deficient Pol γ do not support a direct causal relationship between mtDNA instability and PTP opening. Heart mitochondria from a mouse model displaying tissue-specific increased mtDNA mutations showed a marked inhibition of calcium-induced PTP opening and increased association with the anti-apoptotic protein Bcl-2 [426].

In addition, brain mitochondria from the mutant Pol γ mice sequestered Ca^{2+} more rapidly, although Ca^{2+} retention capacity and mitochondrial ΔΨ were unaffected. Altered Ca^{2+} dynamics in brains from these mice was attributed to downregulated expression of CypD, an effect that was mimicked in wild-type brains by adding cyclosporin A [427]. It is noteworthy, however, that high levels of somatic mtDNA mutations did not increase oxidant production or oxidative stress in the mtDNA mutator mice [428,429].

Taken together, these results suggest that while some pathogenic mtDNA mutations make mitochondria more prone to permeability transition, random accumulation of somatic mtDNA mutations either does not affect or renders mitochondria more resistant to Ca^{2+}-induced MPT. However, it should be considered that all cellular models used in the studies mentioned here had the pathogenic mtDNA mutation in high heteroplasmy or in homoplasmy, while the mtDNA mutator mice carry each particular mutation in very low levels. Thus, it is likely that the impact of somatic mtDNA mutations on permeability transition depends heavily on the target and functional consequences of the mutations, and probably also on the relative frequency of pathogenic versus silent mutations.

The possibility that the opening of the PTP could facilitate release of mtDNA fragments should also be considered. Cytosolic and extracellular mtDNA has been identified as an important agonist of the immune system, through activation of multiple pattern-recognition receptors, functioning as a damage-associated molecular pattern (for review, see [430]). Mitochondrial DNA fragments ranging from around 300–1800 base pairs (bp) were detected in the incubation medium of rat liver mitochondria loaded with as low as 50 nmol Ca^{2+}, but not when CsA was added. Large mtDNA fragments (around 10 Kb) were not detected, suggesting that, indeed, the fragments were released through the PTP [431]. In another study using Fe^{2+}/H_2O_2 to induce MPT in isolated rat liver mitochondria, mtDNA fragments of up to 1000 bp were detected in the incubation medium [432]. Interestingly, it was recently demonstrated that pneumolysin, a pore-forming virulence factor from *Streptococcus pneumoniae*, induces mitochondrial calcium influx and loss of mitochondrial ΔΨ resulting in opening of the PTP and mtDNA release without activation of intrinsic apoptosis [433], establishing a direct causal relationship between MPT, mtDNA release and the inflammatory response.

7. Concluding remarks

There is compelling evidence that intra-mitochondrial Ca^{2+} levels signal for the control of both oxidative phosphorylation and redox balance [14]. In this review we focused on the interactions between the mitochondrial Ca^{2+}-transporting system and cell and mitochondrial oxidant production that lead to redox imbalance and opening of the mitochondrial permeability transition pore (PTP). Under the low conductance states, this pore may regulate physiological cell functions, but under severe redox imbalance, PTP activity seems to be a central event in mechanisms of cell death in several metabolic diseases as well as in cancer, drug toxicity, aging and aged-related diseases. In addition, there is evidence that some mutations in the mtDNA favor opening of the PTP and the generation of mtDNA damage after PTP opening has also been demonstrated. It seems that the most challenging perspective on this topic is the uncovering of the links between redox signals and pore formation and function. The progress in this field may contribute to a better understanding of the mechanisms underlying pore activation, shedding light on the role of mitochondria in cell death in the context of a myriad of diseases.

Acknowledgments

The studies from our laboratory reported in this review were funded by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP # 2006/59786-0, # 2011/50400-0, # 2013/07607-8, # 2014/11261-2, # 2017/17728-8), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) (PNPD20131798) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (307681/2014-9). We thank Annelise Francisco for helping with the figures preparation.

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