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Melatonin and Nitric Oxide

Two Required Antagonists for Mitochondrial Homeostasis

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The presence of nitric oxide ($NO^{\bullet}$) in the mitochondria led to analysis of its source and functions in mitochondrial homeostasis. Studies have revealed the existence of a mtNOS isoform with similar features to nNOS, with some post-traslational modifications, although without the typical signal peptide responsible for addressing proteins to mitochondrion. This isoform may account for the physiological production of $NO^{\bullet}$ related to the respiratory control. During inflammatory conditions there is an excess of $NO^{\bullet}$ in the mitochondria responsible for an increase in reactive oxygen and nitrogen species in sufficient amounts to compromise mitochondrial function. These conditions led to the discovery of the presence of an inducible mtNOS isoform with kinetic properties similar to iNOS. Experiments with knockout mice lacking either nNOS or iNOS further confirmed the existence of these two mtNOS isoforms in mitochondria. Although the increase in $NO^{\bullet}$ in sepsis by inducible mtNOS may have important regulatory functions including the redistribution of oxygen into other pathways under hypoxia, it causes the production of excess $NO^{\bullet}$ that is deleterious for the cell. Melatonin, an endogenous antioxidant, regulates mitochondrial respiration and bioenergetics and protects mitochondria from excess $NO^{\bullet}$ by controlling the activity of mtNOS.

Key Words: Melatonin; nitric oxide; mitochondrial nitric oxide synthase; mitochondria; respiratory chain; ATP; oxidative stress; sepsis.

Introduction

Although the presence and effects of nitric oxide ($NO^{\bullet}$) on mitochondrial function have been known from several years, it was not until recently that the presence of a nitric oxide synthase in this organelle was characterized; this enzyme is responsible for the intramitochondrial production of $NO^{\bullet}$. $NO^{\bullet}$ plays several roles in the mitochondria including the regulation of the respiration through the inhibition of the complex IV activity in competition with oxygen (O_2). Depending on its concentration, $NO^{\bullet}$ may reduce oxygen consumption and may serve as a mechanism for oxygen redistribution. In some instances, the consequence of $NO^{\bullet}$ is the production of free radicals, both reactive oxygen (ROS) and nitrogen species (RNS) that may cause irreversible damage to mitochondria. Several lines of evidence suggest that melatonin exerts specific effects on mitochondria, i.e., increasing respiration and ATP synthesis and reducing the production of ROS and RNS. Both, $NO^{\bullet}$ and melatonin are present in the cells at an early stage of phylogeny, as both are also present in very ancient one-cell organisms including bacteria and parasites. When tested, melatonin and $NO^{\bullet}$ often have opposing actions. This opposite relationship also occurs in mitochondria, and, thus, whereas melatonin improves mitochondrial function, $NO^{\bullet}$ depresses respiration. The present review summarizes the role of the melatonin–$NO^{\bullet}$ couple in mitochondrial homeostasis.

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Mitochondrial Nitric Oxide Synthase

Mitochondria play a central role in the control of O_2 consumption and ATP production. The synthesis of ATP involves the dissipation of the proton electrochemical gradient (or proton-motive force, $\Delta\rho$) produced during electron transport through the electron transport chain (ETC) by ATP synthase. This mechanism is referred to as oxidative phosphorylation (OXPHOS). ETC involves four enzymatic complexes located in the inner mitochondrial membrane: NADH ubiquinone reductase or complex I; succinate ubiquinone reductase or complex II; ubiquinol cytochrome *c* reductase or complex III; and cytochrome *c* oxidase or complex IV. It is now accepted that the redox energy derived from the electron transport across the ETC is stored in the $\Delta\rho$, which comprises two components: the membrane potential ($\Delta\psi_m$) which accounts for the 80% of $\Delta\rho$, and the transmembrane pH gradient ($\Delta\rho H$) *(1)*. The role of the ETC is to reduce O_2 to water by a four-electron reduction of the former at complex IV. However, the ETC work does not reach 100% efficiency and a small amount of O_2 is partially reduced by one, two, or three electrons yielding the superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical (HO^-) *(2,3)*. Among these ROS, HO^- is the most reactive and it may impair mitochondrial function.

Mitochondria also produce RNS, mainly $NO^{\bullet}$ and peroxynitrite ($ONOO^{-}$). The former is produced by mitochondrial nitric oxide synthase (mtNOS), which easily couples with $O_2^{\bullet -}$ yielding $ONOO^{-}$, an extremely reactive compound that irreversibly damages the ETC complexes leading to mitochondrial failure *(4–6)*. Thus, the production of both ROS and RNS, i.e., reactive oxygen and nitrogen species, must be carefully controlled by the antioxidant defense system of the mitochondria to avoid energy failure and death.

How $NO^{\bullet}$ is produced by mitochondria is not totally clarified. It is known that eukaryotic genome sequences do not reveal a specific gene for mtNOS *(7)*. Thus, mtNOS must derive from one of the three NOS isoforms characterized to date. The presence of a mtNOS was initially proposed in 1995 by several groups *(8,9)*. After the first evidence supporting the production of $NO^{\bullet}$ by pure mitochondria, it was not until 1998 that the existence of a mtNOS was identified as the source of intramitochondrial $NO^{\bullet}$ *(10)*. Also, the apparent absence of $NO^{\bullet}$ production in the mitochondria of mice lacking the neuronal but not the endothelial or inducible isoforms of NOS suggested that mtNOS is coded by the same gene as nNOS *(5)*. Because these mice lack the nNOSα variant, it was suggested that mtNOS was related to nNOSα *(5)*. Soon thereafter, others confirmed the relationship of mtNOS to a nNOSα isoform by mass spectrometry, amino acid analysis, molecular weight, pI, and analysis of PCR fragments. The enzyme is processed by a novel post-translational alternative splicing pathway including acylation with myristic acid and phosphorylation at the C terminus *(11,12)*. Whereas the former modification is reversible and is related to its membrane localization, the latter may be related to enzymatic regulation *(11,12)*. It is hypothesized that if ATP and respiratory substrate levels are high, phosphorylation of mtNOS may enhance its activity increasing the production of $NO^{\bullet}$. In turn, $NO^{\bullet}$ may inhibit complex IV, reducing ATP production and O_2 consumption. Thus, the enzyme activity may be linked to O_2 redistribution, which may be important in some pathophysiological conditions *(13)*. The enzyme is localized in the inner mitochondrial membrane and it requires the same cofactors as the cytosolic nNOS. The enzyme uses L-arginine as substrate to produce $NO^{\bullet}$ and L-citrulline. Although the molecular weight of mtNOS and cross-reactivity to monoclonal antibodies against iNOS suggested its relation to iNOS, the mtNOS initially described was related to nNOS owing to its constitutive expression and its main mitochondrial membrane localization *(12)*.

The existence of other NOS isoforms in the mitochondria has been proposed. Some studies suggested the presence of an eNOS isoform, but attempts to identify mtNOS as an eNOS isoform were inconclusive. Similarly, although mtNOS protein was identified with both anti-nNOS and anti-iNOS antibodies in Western blot experiments, the presence of an iNOS isoform in the mitochondria was rejected. A careful analysis of these experiments showed that the characterization of mtNOS was followed using mitochondrial preparations from tissues of normal animals. Because under these conditions iNOS is not expressed or is expressed at very low level, the possibility that an iNOS isoform might be also present in the mitochondrion was not taken into account. Other authors suggested that mtNOS does not correspond to either eNOS, nNOS, or iNOS isoforms, although these studies did not disprove other intramitochondrial sources of $NO^{\bullet}$ such as the conversion of physiological concentrations of NO_2^{-} to $NO^{\bullet}$ *(14)*.

To clarify the presence of other mtNOS isoforms in mitochondria, a series of experiments involving the expression of iNOS were performed. The experimental paradigm consisted in different models of sepsis in rats and mice, induced by either lipopolysaccharide (LPS) injection or cecal ligation and puncture (CLP) *(15,16)*.

Sepsis is a disproportionate inflammatory reaction causing an increase in both the expression and activity of iNOS. Typically, sepsis is accompanied by failed energy generation, which could explain whether an inducible mtNOS exists. The initial data from rats injected with LPS showed that liver and lung mitochondria express a mtNOS that is induced during sepsis *(15)*. The increase in mtNOS protein content was analyzed by Western blot using antibodies against nNOS and iNOS. Two mtNOS isoforms were detected with these antibodies. The mtNOS isoform detected with anti-nNOS antibody was related to the previously characterized mtNOS. In fact, this isoenzyme proved to be Ca^{2+}–calmodulin (CaCaM)-dependent, and its activity was inhibited by the NOS antagonist L-NAME. This isoform of mtNOS, named constitutive mtNOS by analogy to nNOS, is constitutively expressed in the mitochondria and its activity and expression did not change after sepsis. But anti-iNOS antibody also detected another mtNOS isoform with the kinetic characteristics of an iNOS enzyme *(15)*. The activity of this isoform is CaCaM-independent and it is induced during sepsis. However, this inducible mtNOS isoform is also constitutively expressed in normal mitochondria, showing somewhat lower basal activity than constitutive mtNOS. Similar experiments to those showing the lack of mtNOS expression in nNOS-deficient mice were performed in iNOS knockout mice *(16)*. Whereas normal mice show an increase of mtNOS activity after sepsis in mitochondria from several tissues including diaphragm, heart, and skeletal muscle, iNOS-deficient mice did not exhibit detectable inducible mtNOS activity in either control or septic mice (Fig. 1).

Two main findings derived from these studies. First, constitutive and inducible mtNOS derive from nNOS and iNOS genes, respectively. Second, normal mitochondria produce $NO^{\bullet}$ from two mtNOS. These data also explain why mtNOS was identified with antibodies against iNOS and nNOS *(7,15,17)*. The increase in mtNOS activity after LPS has been also documented by other workers in heart and diaphragm mitochondria *(18)*. However, these authors did not differentiate between constitutive and inducible mtNOS isoforms in terms of their dependency of Ca^{2+}.

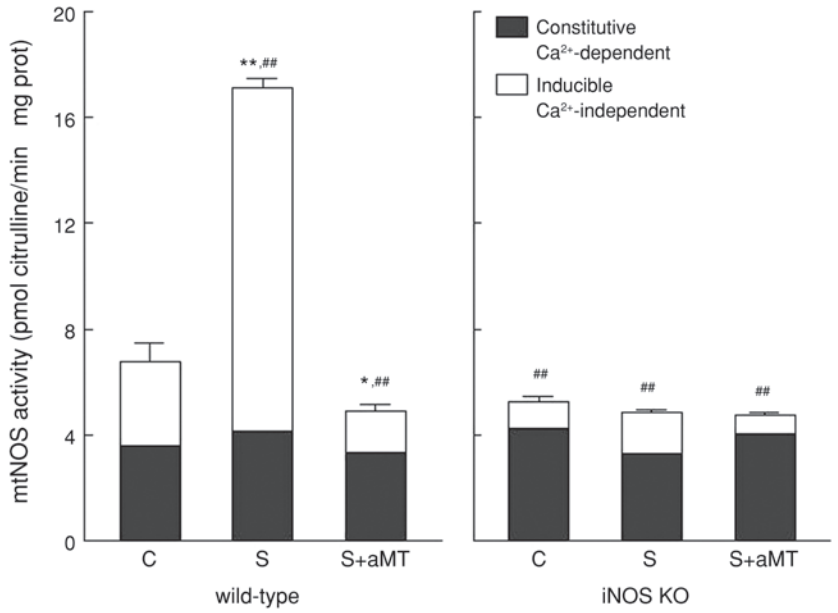

Fig. 1. Changes in constitutive (Ca^{2+}-dependent) and inducible (Ca^{2+}-independent) mtNOS isoforms in diaphragm mitochondria from wild-type (left) and iNOS knockout mice (right). *Left*: Both enzymes are expressed in control mitochondria. Sepsis, however, significantly increases the activity of the inducible isoform without changes in the constitutive one. Treatment with melatonin (aMT) decreased the activity of the sepsis-induced mtNOS below basal levels, without affecting the constitutite mtNOS. *Right*: The inducible mtNOS isoform was almost undetectable in iNOS deficient mice. Neither sepsis nor melatonin treatment modified inducible mtNOS. C, control group; S, sepsis group; S+aMT, sepsis+melatonin group. $^{*}p < 0.01$ vs control; $^{\#\#}p < 0.001$ vs constitutive isoform.

The existence of an inducible isoform of mtNOS may explain some aspects of the inflammatory process, which reduce energy production. Whereas both mtNOS isoforms basally produce $NO^{\bullet}$ in sufficient amounts to regulate respiration, the induction of mtNOS during inflammation generates an excess of $NO^{\bullet}$, which inhibits cytochrome *c* reductase and NADH CoQ oxidoreductase *(19)* leading to a reduction in the electron transfer. The impairment of the respiratory chain by elevated $NO^{\bullet}$ increases the production of $O_2^{\bullet -}$, which in turn reacts with $NO^{\bullet}$ yielding $ONOO^{-}$, a potent prooxidant and cytotoxic agent *(20)*. $ONOO^{-}$ irreversible impairs the respiratory chain and decreases the efficiency of the OXPHOS, leading to energy depletion and cell death *(19,21)*. But $ONOO^{-}$ may also play a protective role in mitochondria, since it induces Ca^{2+} release and subsequent mtNOS deactivation *(22)*. However, this mechanism only accounts for the mitochondrial nNOS isoform that is Ca^{2+}-dependent, but not for inducible mtNOS.

The functional demonstration of mtNOS isoforms has been based on several different approaches including the conversion of oxyhemoglobin to methemoglobin and the conversion of L-^{3}H-arginine to L-^{3}H-citrulline. With each of these methods, incubation of the samples with NOS antagonists caused a complete block of mtNOS activity. A third methodological approximation was the determination of the mitochondrial $NO^{\bullet}$ production from individual mitochondria using voltammetry. These results further supported the presence of a mtNOS *(5)*. The study also showed that mitochondria from nNOS knockout mice did not produce $NO^{\bullet}$. Again, the lack of an adequate experimental paradigm such as sepsis prevented the detection of an inducible mtNOS. The radiometric method used for measuring mtNOS, i.e., the conversion of L-^{3}H-arginine to L-^{3}H-citrulline, may have accounted for some errors in $NO^{\bullet}$ quantification, especially when tissues containing arginases are used. For example, in liver components of the urea cycle in mitochondria can also perform this conversion without $NO^{\bullet}$ synthesis. Moreover, NOS antagonists including L-NAME and several others also inhibit arginase activity *(23)*, and arginases are also induced by LPS *(24)*. However, tissues such as heart, diaphragm, and skeletal muscle do not possess a urea cycle and they showed induction of mtNOS after sepsis. More importantly, the mtNOS induction is absent in iNOS knockout mice, further supporting the presence of a mitochondrial inducible mtNOS unrelated to any arginase activity *(16)*.

The main difficulty in demonstrating mtNOS (either constitutive or inducible forms) in the mitochondria, however, is the degree of mitochondrial purity. In fact, during mitochondrial preparation and depending on the tissue used, some degree of contamination from other subcellular structures always exists. Some of these tissues including heart, muscle, and liver are extensively vascularized and thus contain endothelial cells with large amounts of eNOS, whereas liver contains Kupffer cells, the endogenous macrophages that possess iNOS *(7)*. Mouse cardiomyocytes yield an enriched mitochondrial preparation because they contain 40% mitochondria and only 1% sarcoplasmic reticulum *(25)*, suggesting a high ratio of mitochondria to contaminating orga-

nelles in these preparations. Studies to identify mtNOS as nNOS were carried out in mitochondria preparations with purity varying from 40 to 90% *(5)*. In these preparations, however, the production of mitochondrial $NO^{\bullet}$ was essentially constant, suggesting the lack of significant contamination *(5)*. To characterize the inducible mtNOS was carried out in mitochondria prepared with the same degree of purity including Percoll gradient *(5)*. The crude mitochondrial pellet was obtained after 10,000*g* instead of 100,000*g*, because the former ensures that significantly smaller microsomes are not sedimented *(26)*. It is known that NOS released from membranes in the purification procedure can adhere to mitochondrial membranes during Percoll purification, because this preparation contains up to 4% contamination from other cellular components *(10)*. This contamination mainly accounts for nNOS (and perhaps eNOS) but not for iNOS, which is a soluble enzyme. Also, some reports suggest that iNOS binds non-specifically to membranes even though it contains no acylation residues *(7)*. To ensure the lack of cytosolic iNOS contamination, an exhaustive analysis of the mitochondria samples revealed undetectable NOS activity and $NO^{\bullet}$ levels in the supernatant at the last step of mitochondrial purification *(15,16)*. Finally, the basal activity of inducible mtNOS was similar in different tissues such as lungs, liver, heart, diaphragm, and skeletal muscle.

These data, and the lack of mtNOS induction after sepsis in knockout mice, strongly support the existence of a inducible mtNOS isoform derived from the iNOS gene. The experiments in which mtNOS was identified as an nNOS isoform were always performed under basal conditions, and none included experiments measuring the Ca^{2+}-independent activity of mtNOS. Sepsis is a good model to clarify the roles of each of the mtNOS isoforms in mitochondrial physiology. Thus, it seems that mtNOS types may depend on the tissue of origin and perhaps the species of animal. This means that regulation of mtNOS can vary between tissues *(7,15)*. The question is whether mitochondria contain one or multiple isoforms of NOS. We feel that the constitutive nNOS-derived form controls basal respiration and the inducible iNOS-derived form controls O_2 redistribution.

Nitric Oxide and Mitochondrial Bioenergetics

What is the function of $NO^{\bullet}$ in mitochondria? A previous consideration was that, although the mitochondria are a source of $NO^{\bullet}$ *(10)*, the highly diffusible nature of $NO^{\bullet}$ makes it possible that this gas could be produced extramitochondrially and still regulate mitochondrial function. Thus, it is not essential that $NO^{\bullet}$ be produced near its site of action. $NO^{\bullet}$ is a RNS, with a hydrophobic nature and with low reactivity. These characteristics account for its relatively long half-life (4–6 s) and its high diffusibility, which allows it to reach other cells in the vicinity where it displays autocrine and paracrine properties *(27)*. It was recently shown that the reaction catalyzed by cytochrome *c* oxidase through its peroxynitrite reductase activity is the major pathway for $NO^{\bullet}$ catabolism in mitochondria; this yields nitrite (NO_2^-) *(28)*. Also, cytochrome *c* oxidase has $NO^{\bullet}$ oxidase activity and produces $ONOO^-$. Consequently, a mitochondrion respiring normally may not only suppress $ONOO^-$ formation but also detoxify small amounts of it.

Increasing evidence suggests that $NO^{\bullet}$ plays a central role in mitochondrial bioenergetics through the modulation of the oxygen consumption. $NO^{\bullet}$ is a reversible inhibitor of the cytochrome *c* oxidase *(21)*. Under physiological conditions a mitochondrion has a $[O_2]/[NO]$ ratio of 500–1000, which competitively inhibits cytochrome *c* oxidase by 16–26% *(29)*. Cytochrome *c* oxidase is the terminal enzyme of the mitochondrial respiratory chain and is responsible for 90% of the cellular oxygen consumption. At this level, oxygen is reduced to water, a mechanism coupled to the pumping of protons across the inner mitochondrial membrane. Because $NO^{\bullet}$ competes with O_2 at complex IV, increases in $NO^{\bullet}$ levels prevent the enzyme from using any available O_2, thus causing transient hypoxia *(30)*. This mechanism is especially important in some conditions in which the redistribution of O_2 may be critical for cell survival. Because the constitutive mtNOS is related to the rapid, transient Ca^{2+}-dependent $NO^{\bullet}$ production, uptake of Ca^{2+} by respiring mitochondria increases both $NO^{\bullet}$ and $ONOO^-$, the latter being responsible for Ca^{2+} release through the pyridine nucleotide-dependent pathway *(22)*. This mechanism may be related to the deactivation of the constitutive mtNOS, although it is not applicable to the inducible mtNOS which is Ca^{2+}-independent.

Under physiological conditions, the production of $NO^{\bullet}$ by mitochondria is related to the control of respiration through the reversible inhibition of complex IV. This is a transient inhibition due to the short half-life of the $NO^{\bullet}$ produced by a constitutive mtNOS. However, $NO^{\bullet}$ produced by the inducible mtNOS is sustained, and it is probably related to a extended inhibition of cytochrome *c* oxidase. Thus, the modulation of O_2 consumption by endogenous $NO^{\bullet}$ in normal mitochondria is transient and reversible as long as $NO^{\bullet}$ is generated in small quantities *(22)*. Long-term regulation of respiration and O_2 redistribution may be related to inducible mtNOS. The basic mechanism may be that $NO^{\bullet}$ reduces O_2 consumption by cells closest to blood vessels, allowing O_2 to reach cells with lower PO_2 *(22,30–32)*. This effect, along with the vasodilatatory action of $NO^{\bullet}$, may increase O_2 in hypoxic areas in sufficient amounts to produce the ATP that is needed for metabolism *(31)*. A negative consequence of $NO^{\bullet}$ is the reduction in ATP production and increase ROS/RNS production and nitration of critical proteins *(31)*.

The $NO^{\bullet}$-dependent inhibition of respiration may also maintain a reduced state of the respiratory chain carriers that may react with O_2 partially reducing it to ROS *(32)*. Moreover, because mtNOS requires O_2 as a substrate, some $O_2^{\bullet -}$ is also generated together with $NO^{\bullet}$. The rapid reaction between $NO^{\bullet}$ and $O_2^{\bullet -}$ produces $ONOO^-$ as a consequence of

mtNOS activity. $ONOO^-$ is formed from the diffusion-controlled reaction between $NO^\bullet$ and $O_2^{\bullet-}$ ($k = 10^{10}\ M^{-1}\ s^{-1}$). $ONOO^-$ is in equilibrium with peroxynitrous acid and both are strong oxidizing species in vivo directly or indirectly by secondary decomposition to free radicals after interaction between $NO^\bullet$ and CO_2, including carbonate ($CO_3^{\bullet-}$) and nitrogen dioxide ($^\bullet NO_2$) radicals, and to a lesser extent, $HO^\bullet$. Other alternative sources for $ONOO^-$ in the mitochondria include its diffusion from extramitochondrial compartments; its half-life is calculated to be 10 ms *(6)*. Intramitochondrial production of $ONOO^-$ due to reaction between $NO^\bullet$ and $O_2^{\bullet-}$ also occurs *(6)*. Considering this, the $ONOO^-/CO_2$ pathway is important in the mitochondria, because these are the main organelles producing CO_2. However, because $O_2^{\bullet-}$ diffusion is limited, $ONOO^-$ should be produced at the sites of $O_2^{\bullet-}$ generation, which is largely formed toward the matrix and to a lesser extent in the intermembrane space *(33)*. In any case, $ONOO^-$ reacts avidly with biomolecules resulting in nitration and/or nitrosation of proteins critical for mitochondrial physiology *(6)*.

The interaction of $ONOO^-$ with ETC is different from that of $NO^\bullet$. At normal levels, $NO^\bullet$ seems to reversibly interact with complex IV by competing with O_2. However, $ONOO^-$, but not $NO^\bullet$, reacts with complexes I–V irreversibly, inactivating them *(33)*, as they have various oxidant sensitive sites. The inhibition of complex I- and II-dependent respiration by $NO^\bullet$ thus suggests that $ONOO^-$ mediates these effects *(6)*. $ONOO^-$ also inactivates and nitrates complex V, a finding also found with high $NO^\bullet$ levels. However, complex IV-dependent respiration is not influenced by $ONOO^-$. RNS may nitrate cytochrome *c* and perhaps induce its release, thereby initiating the apoptotic cascade *(33)*. The effect of $ONOO^-$ on permeability transition opening may be also related to its interaction with thiols in the adenosine nucleotide translocator and/or with the voltage-dependent anion channel, both protein complexes taking part in the permeability transition pore. Other membrane-associated proteins including creatine kinase, and nicotinamide nucleotide transhydrogenase can be oxidized and/or nitrated by $ONOO^-$. The difusibility of $ONOO^-$ allows it to react with matrix proteins such as aconitase, magnesium superoxide dismutase (MnSOD), glutathione peroxidase (GPx), and glutathione itself *(34)*. Furthermore, GPx may catalyze the two-electron reduction of $ONOO^-$ to nitrite *(35)*. Also GSH may react directly with $ONOO^-$ in a two-electron oxidation yielding GSSG. Thus, owing to the action of $ONOO^-$, two main antioxidants, GPx and GSH, are reduced. Because GSH also functions to close the PTP *(6)*, this is another pathway by which $ONOO^-$ may induce permeability transition opening and apoptosis. Therefore, several intramitochondrial pathways account for the deleterious RNS actions leading to mitochondria energy failure and eventually apoptosis.

A series of experiments further clarify the relationships between $NO^\bullet$, ROS/RNS, and mitochondria. In pathophysiological situations such as irradiation, it was reported that mtNOS plays a deleterious role. In fact, irradiation opens the mitochondrial permeability transition pore, increasing matrix Ca^{2+} and activating mtNOS *(25)*. $NO^\bullet$ inhibits complex IV increasing $O_2^{\bullet-}$ production at the CoQ level. In this situation MnSOD may produce larger amounts of H_2O_2. Two main situations may result: the levels of GPx may be insufficient, resulting in the formation of highly toxic $HO^\bullet$ and tissue damage, or a deficit in glutathione reductase (GRd) activity may occur because this enzyme is sensitive to oxidative damage and it may be destroyed by increased levels of either $O_2^{\bullet-}$ or H_2O_2. In the latter situation, not enough GSSG is recycled to GSH, and mitochondria lack GSH to further transform H_2O_2 to water *(25)*. In experiments with sepsis, LPS administration to rats resulted in a 40-fold increase of inducible mtNOS activity, with a parallel increase in the intramitochondrial levels of $NO^\bullet$. These increases are compatible with a sustained inhibition of complex IV *(15)*. Similar results were found with septic mice, whereas iNOS deficient mice did not show changes in the activity of mtNOS or $NO^\bullet$ levels after CLP-induced sepsis *(16)*. The newly formed $ONOO^-$ may react with tetrahydrobiopterin (BH4), yielding the $BH_3^\bullet$ radical. This leads to the dissociation of mtNOS and a parallel increase in $O_2^{\bullet-}$ *(36)*. Consequently, there is a rise in both $O_2^{\bullet-}$ and $ONOO^-$. The maintenance of high levels of $ONOO^-$ leads to an impairment of the ETC, with a decrease in ATP.

If inducible mtNOS exists, what is its role in mitochondrial physiology? We suggest that, similar to cytosolic iNOS, the induction of mtNOS may have a protective function. Depending on the $O_2/NO^\bullet$ ratio, respiration may vary between physiological values. During an inflammatory condition such as sepsis, the increased $NO^\bullet$ reduces respiration, thereby lowering O_2 consumption. This mechanism permits O_2 redistribution toward other cells or areas with urgent requirements for O_2 to prevent them from becoming hypoxic. Within certain limits, the reductase activity of cytochrome *c* oxidase may detoxify $ONOO^-$ reducing ETC damage *(28)*. Above a particular threshold, however, the excessive $NO^\bullet$ in the mitochondria produce higher amounts of $ONOO^-$ than complex IV can detoxify, leading to an irreversible damage of the complexes of the respiratory chain. Under these conditions the beneficial effect of the $NO^\bullet$ becomes harmful, leading to mitochondrial failure, energy loss, and, in the case of sepsis, multiorgan failure and cell death.

A last consideration regarding the role of $ONOO^-$ and its effects in mitochondria should be noted. It was shown that $ONOO^-$ increases proton leakage *(37)*, perhaps due to the modification of cysteine and tyrosine residues in the uncoupling proteins (UCPs) *(37)*. UCP-dependent mitochondrial uncoupling dissipates $\Delta\psi_m$, resulting in a lower rate of $O_2^{\bullet-}$ production. Under these conditions, MnSOD converts $O_2^{\bullet-}$ to H_2O_2, which may be related with the maintenance of the redox tone of the mitochondria that may serve signaling purposes. In sepsis, parallel to the upregulation of iNOS

is also an increased UCP expression *(37)*, which, in turn, inhibits $O_2^{\bullet -}$ production thus preventing excessive $ONOO^-$.

The debate over whether activation of mtNOS is beneficial or detrimental for cell function should be related to the type of mtNOS that is activated, to the tissue and its condition. Furthermore, a controversy exists regarding the concentrations of $NO^\bullet$ used for complex IV inhibition in experiments in vitro and the physiological production of $NO^\bullet$. Experiments performed with well-controlled physiological $NO^\bullet$ concentrations fail to show inhibition of respiration *(7)*. The authors concluded that the published rates of $NO^\bullet$ production by mtNOS (1.7 nmol/min·mg protein) are not physiological because at this concentration $NO^\bullet$ should produce a strong inhibition of complex IV *(7)*. Thus, the values for $NO^\bullet$ may be greatly overestimated. For example, in pathological myocardium mitochondria, $NO^\bullet$ may limit myocardial contractility protecting the heart. The negative consequence is the reduction of ATP, cardiac hypertrophy, and heart failure *(25)*. As a general rule, after the activation of the constitutive mtNOS, the production of $NO^\bullet$ may be sufficient to partially inhibit respiration. However, when the inducible mtNOS is the isoform activated, then the production of $NO^\bullet$ is several times higher than in the former, and these levels of $NO^\bullet$ may strongly inhibit complex IV and respiration, leading to a redistribution of O_2 and, in some cases, to mitochondrial energy loss and cell death. Thus, the debate regarding mtNOS should be addressed with the further characterization of the two mtNOS isoforms, their mechanisms of post-translational modifications and mitochondrial importation, and their role in physiological conditions in which, apparently, the two isoforms are present.

Melatonin and Nitric Oxide in Mitochondrial Homeostasis

After the first report suggesting the antioxidant role for melatonin *(38)*, a large series of experiments both in vivo and in vitro further demonstrated its antioxidant potential *(39–47)*. Melatonin not only may scavenges ROS and RNS, it also increases the expression and activity of some antioxidant enzymes, while reducing the activation of prooxidant enzymes *(48–50)*.

From a phylogenetic point of view, melatonin is an ancient molecule. It is a highly conserved indoleamine derived from tryptophan, and it is present in all living beings, from one-cell organisms to mammals. The presence of melatonin in cyanobacteria, unicell algi, and parasites such as *Trypanosoma cruzi* supports its early presence in the origin of life *(51,52)*. In mammals, melatonin is synthesized by the pineal gland in a circadian manner and it is released to blood, where its concentration may increase to 0.5 n*M* *(53)*. It is currently known that melatonin is also produced by many organs/tissues *(54)*. At least in some of these organs, melatonin levels are several times higher than those found in blood. These different sources of melatonin may be related to the different actions of the indoleamine. Interestingly, melatonin is also concentrated by subcellular compartments including nucleus and mitochondria, the latter showing 100–200 times more melatonin than the cytosol *(55)*. Melatonin also interacts with lipid bilayers and stabilizes mitochondrial inner membranes *(56)*. Among other considerations, the high levels of melatonin in some tissues and bodily fluids generally supports its antioxidant and free-radical-scavenging capacity. Moreover, although the pico- to nanomolar concentrations of melatonin in blood would generally not provide strong free-radical-scavenging activity, the concentrations are sufficient for genomic regulation of the activities of several pro- and antioxidant enzymes *(42,49,50)*. In fact, the indoleamine reportedly reaches a concentration in the cell nucleus of 1 n*M*, sufficient to saturate nuclear receptors that possess a K_D near to 200 p*M* *(57)*.

The first important feature of melatonin is its ability to detoxify a series of ROS including singlet oxygen, $HO^\bullet$, and H_2O_2 *(47)*. Melatonin has the benefit that the products of detoxification, N^1-acetyl-N^2-formyl-5-methoxy kynurenamine (AFMK) and *N*-acetyl-5-methoxy kynurenamine (AMK), are also efficient antioxidants *(58–60)*. This led to the proposal of a "cascade of antioxidants" regarding the consequences of melatonin's actions. Also, neither melatonin nor its metabolites exert prooxidant effects as occurs with other antioxidants such as vitamins C and E. The second important feature of melatonin is its ability to increase the expression and activity of enzymes directly involved in antioxidant protection. These enzymes include GPx and GRd, SOD, and catalase (CAT) *(42,49)*. Furthermore, melatonin directly increases the *de novo* synthesis of GSH by stimulating the activity of γ-glutamyl-cysteine synthetase. The combination of the direct antioxidant actions of melatonin plus its regulation of the antioxidant enzymes accounts for many of the protective actions of melatonin against oxidative stress.

Melatonin also scavenges RNS including $NO^\bullet$ and $ONOO^-$, and reduces the amount of $NO^\bullet$ generated by NOS *(47,48, 50)*. Melatonin binds CaCaM with high affinity, avoiding the activation of the CaCaM-dependent enzymes, including nNOS *(61)*. One of the melatonin metabolites, AMK, and synthetic analogs structurally related to AMK also bind CaCaM and reduce nNOS activity in a dose-dependent manner *(61)*. These data, together with other experiments using electrophysiological techniques *(62)*, suggest that the effects of melatonin on NOS activity are mainly produced through its metabolite AMK *(60)*. Another series of experiments have documented the inhibition of the expression and activity of iNOS by melatonin *(15,50)*. Whether the effects of melatonin on iNOS depend on its metabolites or are a direct effect of the indoleamine remain to be tested. In any case, the reduction of iNOS by melatonin leads to a significant reduction of $NO^\bullet$ levels, reducing the toxic effects of this gas *(15,50)*.

The above summarized data regarding the antioxidant effects of melatonin against ROS/RNS, the observation that mitochondria concentrate melatonin, and the fact that mitochondria are the main source of ROS/RNS in the cell, suggest a physiological role for the indoleamine in the mitochondrial homeostasis *(63,64)*. To assess the functional role of melatonin in the mitochondria, a series of experiments were performed on normal mitochondria and in diseases involving mitochondrial failure.

In vivo experiments have shown that melatonin administration increases the activity of the complexes I and IV of the ETC in mitochondria from brain and liver *(65)*. The effect was time-dependent and in agreement with the half-life of the indoleamine. In animals treated with ruthenium red, a compound that blocks Ca^{2+} channels and reduces the activity of the mitochondrial ETC, melatonin administration counteracted the toxicity of this compound, normalizing the activity of the respiratory chain *(65)*. These experiments led to in-depth examination of the effects of melatonin on mitochondrial function. The initial experiments were conducted with normal mitochondria prepared from brain and liver and incubated in vitro. In these mitochondria, incubation with 1–100 n*M* melatonin increased the basal activity of the complexes I and II in a concentration-related manner, increasing the ATP production *(55)*. Other experiments were performed on mitochondria incubated in the presence of *t*-butyl hydroperoxide (BHP), a prooxidant and inductor of hydroperoxides which induce elevated mitochondrial oxidative stress with inhibition of the respiratory chain. The simultaneous incubation with 1–10 n*M* melatonin partially counteracted the inhibition of complexes I and IV induced by BHP, and at 100 n*M* melatonin totally normalized the mitochondrial function and restored ATP production *(55,66)*. But BHP also induced a strong oxidative stress and depleted mitochondria GSH and inhibited both GPx and GRd activities. Under these conditions, mitochondria are unable to resist oxidative stress because they have little GSH and cannot recycle it from GSSG. In this situation, incubation with 100 n*M* melatonin totally counteracted the effects of BHP, recovered the GSH pool, and restored GPx and GRd activities *(55)*. It is interesting to note that other antioxidants including *N*-acetylcysteine and vitamins C and E, up to 1 m*M*, did not exert a significant effect on the mitochondria damaged by BHP *(55)*. Thus, melatonin counteracted oxidative stress, enhanced the activity of the ETC complexes I and IV, and increased the production of ATP. The effect of 1–10 n*M* melatonin on respiratory complexes I and IV was dose-dependent; this resulted in a further increase above the basal values at higher concentrations of the indoleamine, suggesting a direct effect of melatonin on mitochondrial function. The interaction between melatonin and complex I was further analyzed by blue-native polyacrylamide gel electrophoresis. This method permitted the detection of the increase in complex I activity induced by melatonin directly, suggesting an interaction between the indoleamine and this enzyme *(66)*. Regarding complex IV, a series of titration curves in the presence of a selective inhibitor of this complex, cyanide, showed observe that melatonin counteracted, in a dose-dependent manner, the inhibition of the enzyme *(66)*.

The molecular characteristics of melatonin account for its described effects on mitochondria. Melatonin is a highly lipophilic molecule that crosses cell membranes reaching subcellular compartments including the mitochondria *(66)*, where it is seemingly concentrated. Melatonin also interacts with lipid bilayers and stabilizes mitochondrial inner membranes. Moreover, melatonin seems to have a high redox potential *(67)*, suggesting that it may interact with the respiratory complexes and donate electrons improving the electron flow.

As discussed above, normal mitochondria produce both $NO^{\bullet}$ and $ONOO^{-}$. Whereas the former is used for respiratory control, the latter is toxic for the mitochondria and it is normally scavenged. Because melatonin scavenges $NO^{\bullet}$ and $ONOO^{-}$, the levels of melatonin in normal mitochondria may participate controlling the levels of these RNS. Because $NO^{\bullet}$ inhibits and melatonin increases the activity of complex IV, these molecules may act together in regulating the respiration according to the cell requirements. Melatonin also inhibits both the activities of nNOS and iNOS and, thus, it is likely that the indoleamine also participates in the regulation of mtNOS. To assess this possibility, the effect of melatonin on mtNOS and mitochondrial $NO^{\bullet}$ content in both basal and septic conditions was assessed. Under basal conditions both inducible and constitutive mtNOS isoforms are expressed in mitochondria of liver and lungs, and $NO^{\bullet}$ levels into the mitochondria in these conditions depend on both isoforms activity *(15)*. Western blot analysis of control mitochondria with both anti-iNOS and anti-nNOS antibodies revealed two mtNOS isoforms being constitutively expressed. After induction of sepsis with LPS, Western blot analysis revealed an increase in the mtNOS protein content identified with anti-iNOS antibody, whereas mtNOS protein content identified with the anti-nNOS antibody remained unchanged *(15)*. The increase in the inducible mtNOS isoform was accompanied by a parallel increase in $NO^{\bullet}$ levels, and a reduction in the activity of the respiratory complexes I and IV *(15)*. Melatonin administration to septic rats prevented the induction of mtNOS and the increase in $NO^{\bullet}$, and the respiratory chain activity recovered its normal activity. The septic condition after LPS administration was accompanied by multiorgan failure, with significant effects on the liver, kidneys, lungs, and metabolism. Mitochondria improvement after melatonin administration was followed by a normalization of the function of these organs, avoiding the death of the septic animals *(15)*.

Sepsis is a condition caused by ROS/RNS and these free radicals strongly influence mitochondria. The remaining question is the degree to which ROS/RNS affect mitochondria and whether melatonin can counteract this category of

radicals to the same degree. To answer these questions, experiments in knockout mice lacking the iNOS gene were performed. Mitochondria from different tissues of normal mice including liver, heart, diaphragm, and skeletal muscle typically exhibit the two isoforms of mtNOS constitutively expressed. Induction of sepsis with either LPS administration of CLP caused a significant increase in the inducible isoform of mtNOS in mitochondria, whereas the constitutive isoform remained unchanged *(16)*. The levels of $NO^{\bullet}$ also increased significantly after CLP. However, mice lacking the iNOS gene did not show any modification in the mtNOS activity after sepsis, and the levels of $NO^{\bullet}$ remained at the basal level *(16)*. The data suggest that in inflammatory states such as sepsis the main source of intramitochondrial $NO^{\bullet}$ is the inducible isoform of mtNOS. The increase in $NO^{\bullet}$ levels was accompanied by a 30–50% reduction in the activity of the four complexes of the ETC. The respiratory chain damage is associated with the inducible mtNOS because iNOS deficient mice do not show changes in respiratory chain activity *(16)*. Mice lacking the iNOS gene exhibited control $NO^{\bullet}$ levels lower than wild-type mice, suggesting that, under physiological conditions, the intramitochondrial pool of $NO^{\bullet}$ depends on both mtNOS isoforms. Under these conditions, the constitutive mtNOS isoform provides about 68% of the mitochondrial $NO^{\bullet}$ pool, with the remaining 32% coming from inducible mtNOS. The reduction in the mitochondrial $NO^{\bullet}$ pool in iNOS-deficient mice was sufficient to increase almost twofold the activity of the complexes III and IV in control mitochondria. These data indicate that a partial inhibition of complexes III and IV by the $NO^{\bullet}$ derived from the inducible, Ca^{2+}-independent mtNOS could be present in those studies in which the activity of mtNOS is blocked with EGTA to remove the Ca^{2+}-dependent mtNOS *(16)*.

Melatonin administration to septic mice documented a similar effect to that observed in rats treated with LPS, i.e., an inhibition of the inducible mtNOS and a normalization of $NO^{\bullet}$ levels *(15,16)* (Fig. 1). Melatonin also increased the activity of the four complexes of the ETC in these mice. Interestingly, melatonin administration had no effect on mtNOS activity and $NO^{\bullet}$ levels in mice lacking the iNOS gene *(16)*. These findings suggest that melatonin acts when mitochondrial function is impaired. Septic mice also showed a significant oxidative stress reflected by an increase in lipid peroxidation and the GSSG/GSH ratio, and reduction in the activity of the redox enzymes GPx and GRd *(15)*. These markers of oxidative stress were also counteracted by melatonin administration. iNOS deficient mice, however, did not show changes in either the GSH pool or GPx and GRd activities after sepsis *(16)*.

These experiments revealed that the lack of the iNOS gene protects mitochondria against sepsis. The data also suggest that the inducible component of mtNOS is responsible for the sustained increase in $NO^{\bullet}$ during inflammation. Finally, the results confirm that $NO^{\bullet}$ is a molecule that triggers the intramitochondrial production of ROS including $O_2^{\bullet -}$, $HO^{\bullet}$, and H_2O_2 *(32)*, leading to an $ONOO^-$ increase. In terms of the redox status of the mitochondria, the data obtained with melatonin treatment parallel those found in iNOS-deficient mice. Both conditions prevent mitochondrial oxidative damage during sepsis. Because melatonin reduced the expression and activity of iNOS and mtNOS induced by sepsis *(15,16,50)*, the reduction in the availability of $NO^{\bullet}$ to mitochondria seems to be the primary effect of melatonin.

Concluding Remarks

This review summarized a series of data regarding the presence of $NO^{\bullet}$ in the mitochondrion, its mechanism of production and regulation, and its role in mitochondrial function. Also, we describe the particular relationships between melatonin and $NO^{\bullet}$ as both molecules seem to participate in the regulation of mitochondrial homeostasis, although their actions are opposed. The results presented herein help to enhance understanding of the mechanisms involved in mitochondrial regulation by $NO^{\bullet}$ and they provide experimental evidence for the existence of two mtNOS isoforms with differential functions. Constitutive mtNOS may regulate basal respiration, and inducible mtNOS may contribute to O_2 redistribution. The main characteristics of mitochondria are related to their ability to be both a source and a target of ROS/RNS. Normally, $NO^{\bullet}$ triggers the production of mitochondrial ROS for physiological processes, whereas an increased production of $NO^{\bullet}$ is associated with mitochondrial damage. In the presence of a suitable response of inducible mtNOS under conditions such as sepsis, sufficient amounts of $NO^{\bullet}$ are released displacing the mitochondrial balance toward the production of free radicals. In these conditions, endogenous melatonin levels may be insufficient to counteract the excess of $NO^{\bullet}$, but its exogenous administration efficiently restores normal mitochondrial homeostasis. This is consistent with other reported in vitro and in vivo effects of melatonin *(68–71)*. To summarize, the actions of melatonin related to mitochondrial physiology include (Fig. 2): (a) inhibition of the expression and activity of inducible mtNOS, (b) antagonism of ROS/RNS, (c) restoring the mitochondrial GSH pool, (d) closing the permeability transition pore avoiding apoptosis and reducing the Ca^{2+} influx to mitochondria, (e) enhancing the activity of the respiratory chain complexes, and, finally, (f) it behaves as an electron donor. Additionally, melatonin reduces the toxicity and increases the efficacy of drugs *(40)*. These actions support a role for melatonin as a multi-faceted drug in terms of its pharmacological efficacy.

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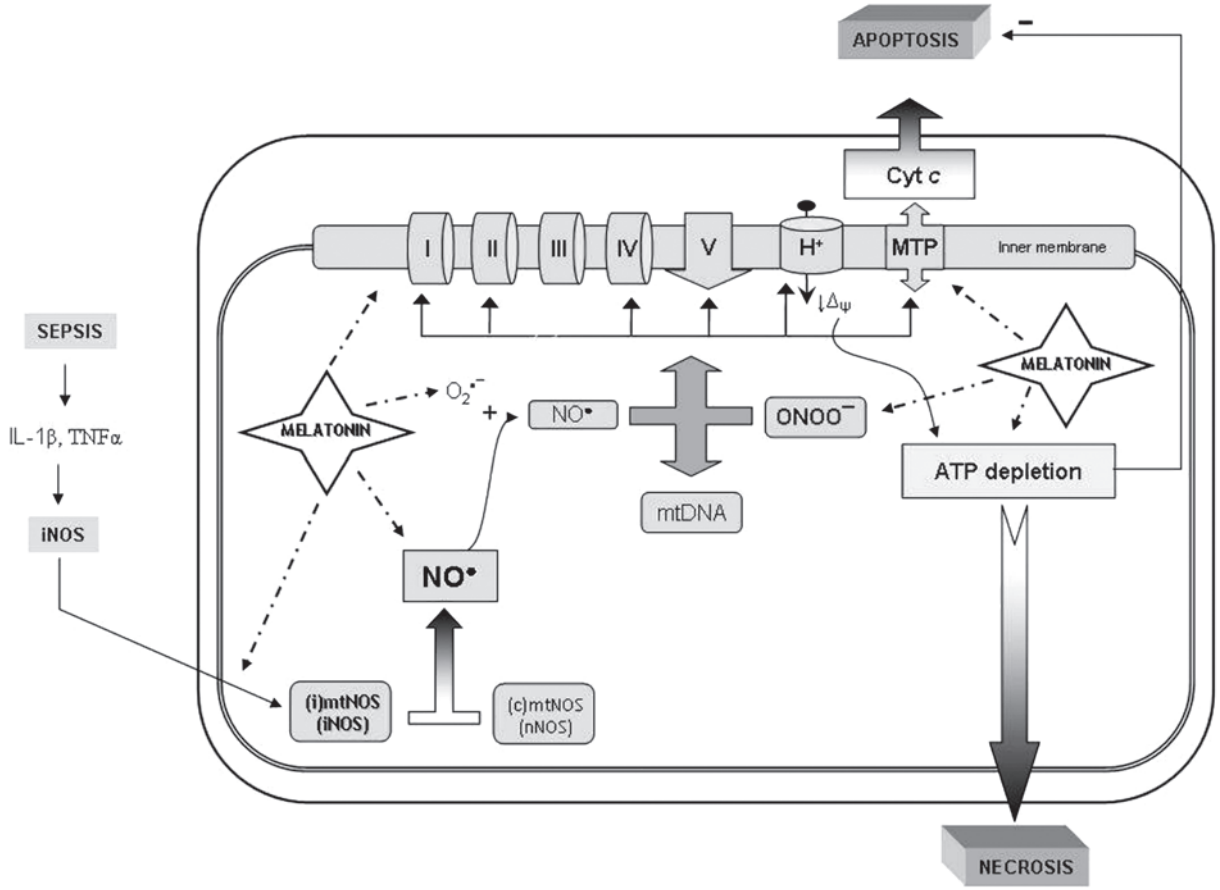

Fig. 2. Schematic representation of the main routes involved in mitochondria damage in sepsis. After sepsis, only the inducible mtNOS isoform increases, producing an excess of $NO^{\bullet}$ that rapidly reacts with $O_2^{\bullet-}$ generating $ONOO^-$. In turn, $ONOO^-$ irreversibly damages the respiratory chain components, decreasing $\Delta\psi_m$ and resulting in reactive oxygen species generation, ATP depletion and cell death. $ONOO^-$ may also directly induce the mitochondrial permeability transition pore (MPT) and cell death. Melatonin administration to septic animals counteracts the induction of mtNOS, scavenges ROS and RNS, increases the activity of the respiratory complexes, and restores $\Delta\psi_m$ and ATP levels. Melatonin also directly closes the permeability transition preventing cell death.

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