Chapter 4
Biochemistry and Physiology of Heart Mitochondrial Nitric Oxide Synthase

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Abstract Heart mitochondria are the major source of reactive oxygen and nitrogen species and play a central role in cell energy provision and signaling. The NO produced by cardiac mtNOS is allowed to interact restrictedly with the co-localized effectors. NO exerts a high affinity, reversible and physiological inhibition of cytochrome *c* oxidase activity. A second effect of NO on the respiratory chain is accomplished through its interaction with ubiquinol-cytochrome *c* oxidoreductase. The ability of mtNOS to regulate mitochondrial O_2 uptake and O_2^- and H_2O_2 productions is named mtNOS functional activity. Several situations, including chronic hypoxia and ischemia-reperfusion, modify heart mtNOS activity or expression. The regulation of heart mtNOS by distinctive mitochondrial environments includes the effects of Ca^{2+}, O_2, L-arginine, NADPH, mitochondrial membrane potential ($\Delta\psi$) and the metabolic states. Together, this enzyme seems to be critical during the adaptation of heart mitochondria to changes in cellular bioenergetics.

Keywords Heart mitochondrial NOS • mtNOS functional activity • Nitric oxide • Membrane potential • Hydrogen peroxide • Mitochondrial complexes I, III and IV

1 Introduction

Mitochondria supply the cells with both the energy and the signals that coordinate cell death and survival [1]. Indeed, heart mitochondria produce the potentially toxic products of the partial reduction of oxygen: superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^\bullet$) and signaling and regulatory molecules, as nitric oxide (NO) and H_2O_2 [2–5]. A fine control of mitochondrial respiration is critical to meet the energy demand of cardiac muscle [6–8]. During the progression

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of heart disease, the loss of mitochondrial function leads to cell injury, cell dysfunction and death [8]. The signaling functions of NO in the heart depend on its production by the specific NO synthase (NOS) isoforms compartmentalized within cellular microdomains, and its interactions with several biomolecules involved in downstream signaling pathways. The strategic localization of heart mitochondrial nitric oxide synthase (mtNOS) in the organelles in charge of cellular energy metabolism allows for a tight control of different processes whereby mitochondria play pivotal roles [9, 10].

2 Nitric Oxide Regulates Mitochondrial Respiration and Superoxide Anion and Hydrogen Peroxide Productions

Even though NO is a highly diffusible molecule, the distances that this free radical reaches are short due to its high reactivity with several species which include heme groups, O_2^- and thiols [9]. As a result, the NO produced by cardiac mtNOS is allowed to interact restrictedly with the co-localized effectors. It is well known that NO activates soluble guanylatecyclase (sGC), which leads to the production of the second messenger 3,5′-cyclic guanosine monophosphate (cGMP) [8]. However, certain functions of NO in signaling and regulation of cardiac function are performed through cGMP-independent pathways including those that involve mitochondria (Fig. 4.1).

First, NO competes with O_2 for the binding site at the binuclear center of cytochrome *c* oxidoreductase or complex IV. This leads to a high affinity and reversible inhibition of the enzyme [11–13]. This effect was unraveled using a mathematical model based in experimental data and described different effects of NO in the mito-

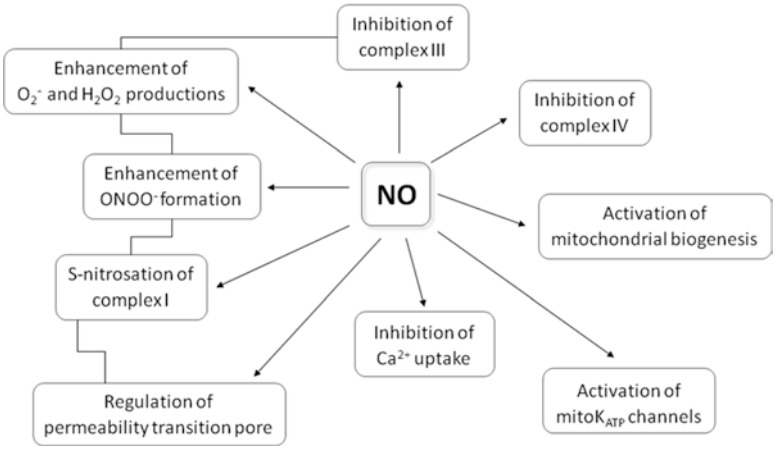

Fig. 4.1 Effects of NO in mitochondria

chondrial metabolic states [14, 15]. The mitochondrial NO steady-state concentrations in tissues, in the 50–350 nM range [6] are in the same range that produces an effective (50 %) inhibition of cytochrome oxidase activity [6, 7, 12–15]. The rate of respiration depends on O_2 concentration and on the O_2/NO ratio [6, 7]. In other words, the biological effect of a given NO concentration will depend on the simultaneous O_2 concentration; a lower PO_2 increases and extends the effects of NO [6, 7]. Additionally, another important effect of NO on mitochondrial respiratory chain is accomplished through the interaction of NO with respiratory complex III, ubiquinol-cytochrome *c* oxidoreductase [16, 17]. The activity of succinate-cytochrome *c* reductase (complexes II-III) was inhibited by about 50 % in the presence of about 1.3 μM NO released from the NO donors S-nitrosoglutathione (GSNO) or spermine-NONOate, while the activity of succinate-Q reductase (complex II) resulted unaffected [17]. These results indicate that NO specifically inhibits complex III activity. Interestingly, complexes II-III activity was also decreased (36 %) when submitochondrial particles (SMP) were incubated with mtNOS substrates and cofactors, suggesting that the inhibition is also produced by endogenous NO. Through its interaction with components of the electron-transfer chain, NO functions not only as a physiological inhibitor of cell respiration, but it also enhances the generation of reactive oxygen species [16, 17] and thereby triggers various mechanisms underlying the survival or death of the cells. A hyperbolic increase in O_2^- and H_2O_2 production rates by heart SMP was observed with a maximal effect at 500 μM GSNO [17]. Moreover, H_2O_2 production by heart coupled mitochondria was increased by 70 % when mitochondria were exposed to the NO donors [17].

The activity of heart mtNOS can be assessed indirectly through the two main effects that NO exert on the mitochondrial respiratory chain functions: the inhibition of O_2 consumption and the enhancement of H_2O_2 production. This mtNOS activity has been termed *mtNOS functional activity* [18] and it is determined by the difference in the rates of O_2 uptake or H_2O_2 production in isolated mitochondria in two different conditions. One of them is the condition where NO steady-state concentration is the highest, *i.e.* in the presence of sufficient L-arginine and SOD, condition in which active O_2 consumption is reduced and H_2O_2 production is enhanced. The second condition is by the contrary, when NO steady-state level is the lowest, condition in which active O_2 consumption is increased and H_2O_2 production is decreased. This latter situation is achieved in the absence of NOS substrates or cofactors and in the presence of a NOS inhibitor or a NO scavenger, *e.g.* oxyhemoglobin. Thus, supplementation of heart mitochondria with L-arginine and SOD decreases the respiration rate by 15–20 %, while supplementation of the mitochondrial preparation with a NOS inhibitor (L-NAME) and hemoglobin increases O_2 consumption by 10–15 %. Concerning H_2O_2 production, the addition of L-arginine and SOD enhances H_2O_2 production by 15–20 % in heart mitochondria, whereas the supplementation of the same preparation with L-NAME and hemoglobin declines H_2O_2 generation by 10–15 %. Thus, the changes in mtNOS functional activity clearly reveal variations in mitochondrial NO production rates and NO steady-state concentration [18]. For instance, the myocardial stunning observed in perfused

rabbit heart after 15 min ischemia and 30 min reperfusion, is characterized by a decreased mtNOS activity and an impaired mtNOS functional activity [19].

3 Other Effects of NO and NO-Derived Species on Mitochondrial Functions

Other effects of NO in mitochondria-cytosol signaling are conveyed via nitrosation of proteins [20]. In nitrosation reactions, NO reversibly reacts with the nucleophilic centers in thiol residues contained within a specific consensus sequence of amino acids [21] of a broad array of low molecular weight compounds or proteins [21, 22]. When mitochondria are treated with NO donors, complex I is S-nitrosated resulting in a significant inhibition of this respiratory complex, an effect that can be reversed by light or reagents containing thiol groups [23]. It has been suggested that a transient reversible inhibition of the mitochondrial electron transfer minimizes ischemia-reperfusion injury, and that the blockade of electron transfer at complex I preserves respiration during reperfusion [24]. Studies have shown that complex I inhibition by nitrosation protects mitochondria during hypoxia and reoxygenation and cardiomyocytes during ischemia-reperfusion [25–27]. Nitric oxide also reacts with O_2^- that is formed by the mitochondrial respiratory chain during normal or pathological oxygen metabolism, yielding $ONOO^-$ [28]. This reaction occurs with a second order rate constant of about 2×10^{10} M^{-1} s^{-1} and its rate is controlled by the diffusion of the reactants [29]. Peroxynitrite influences cardiac contractility and, in some cases, produces effects that are markedly different from those of NO [30, 31]. This NO congener may hinder mitochondrial functions and cause cell death. The switch from reversible inhibition of cellular respiration by NO to the pathological inhibition of mitochondrial function by the NO-derived $ONOO^-$ has been observed in many physiopathological conditions, in which intramitochondrial NO and O_2^- steady-state concentrations are enhanced. Pathological concentrations of NO are likely to affect respiration by mechanisms that are qualitatively different from those observed during reversible physiological regulation. Increased $ONOO^-$ steady-state concentration together with mitochondrial malfunctioning are hallmarks of heart hypoxia-reperfusion injury [19, 30, 31].

In addition, NO may act on other mitochondrial targets. It has been reported that NO increases oxidative phosphorylation efficiency [32], activates mitoK_{ATP} channels [33], regulates mitochondrial matrix pH and Ca^{2+} buffering capacity [34], triggers mitochondrial biogenesis [35] and modulates the mitochondrial permeability transition pore (MPT)) formation [36]. The regulation of mitochondrial Ca^{2+} accumulation by NO may provide means by which mtNOS can influence mitochondrial metabolism as well as survival [37]. Nitric oxide triggers mitochondrial biogenesis in several cell types and tissues, including those obtained from heart, through a cGMP-dependent mechanism [35]. The cell injury and death observed after reoxygenation of the tissue are in accordance with several reports showing that NO can

prevent or accelerate mitochondrial permeability transitions [38]. This depends on whether NO concentrations are physiological or supraphysiological and whether O_2^- is generated simultaneously [39, 40]. In this regard, heart mtNOS plays an important role suggesting relevance of mtNOS in yet another important mitochondrial function.

4 Heart mtNOS Activity and Identity

Different groups have used various experimental approaches and reported the presence of mtNOS in the heart. A summary of the publications is included in Table 4.1. The first study showing the presence of a NOS isoenzyme located in mitochondria used the silver enhanced gold immunolabelling method and showed that about 85 % of the heart mitochondria were positive for the eNOS label [41]. After that, the studies of cardiac mtNOS have used various methods: immunohistochemistry [41, 42], spectrophotometry [19, 39, 43–51], radiometry [52, 53], fluorometry [40, 54–56], chemiluminescence [53] and electrochemistry [57]. Heart mtNOS has been studied

Table 4.1 mtNOS activity or expression in heart mitochondria

Methodology	Sensitivity [NO] (M)	References
Espectrophotometric (HbO_2 oxidation)	$1 \times 10^{-11}/10^{-12}$	Costa et al. [43]
		Boveris et al. [39]
		Saavedra-Molina et al. [44]
		Valdez et al. [45]
		Gonzales et al. [46]
		Zaobornyj et al. [47]
		Fellet et al. [48]
		Boveris et al. [49]
		La Padula et al. [50]
		Zaobornyj et al. [51]
		Valdez et al. [19]
Radiometric ([^{3}H]L-citrulline/[^{14}C] L-citrulline))	1×10^{-7}	Zanella et al. [52]
		Zenebe et al. [53]
Microsensor (porphyrinic and amperometric)	1×10^{-7}	Kanai et al. [57]
Fluorometric (DAF-2, DAF2-DA, DAF-FM)	10^{-9}	Lopez-Figueroa et al. [54]
		Zanella et al. [55]
		Zorov et al. [56]
		Dedkova and Blatter [40]
Chemiluminescence		Zenebe et al. [53]
Immunohistochemistry		Bates et al. [41]
		Hotta et al. [42]

using isolated cardiac mitochondria [19, 39, 43–51] and through functional experiments in which mitochondrial membrane potential-dependent NO production was measured in cardiomyocytes [40, 49]. Kanai and co-workers [57] showed elegantly the presence of a NOS activity in mouse cardiac mitochondria by measuring, in a single individual mitochondrion, the NO production that followed to Ca^{2+} addition to the reaction medium, by using a porphyrinic microsensor. Moreover, Boveris group [19, 39, 43, 45, 47, 49] reported a heart mtNOS activity of 0.8–1.5 nmol NO × min^{-1} × mg $protein^{-1}$ using the oxyhemoglobin (HbO_2) assay. Heart mitochondrial NO production accounts for about 60 % of total cellular heart NO generation, suggesting a central role of the mitochondrially produced NO in cardiomyocytes [51, 58]. Confocal microscopy using fluorescent probes for coupled mitochondria and for NO indicated the presence of NO in mitochondria [40, 55]. Other studies have detected mitochondrial NO release using a direct spin trapping technique with electron paramagnetic resonance spectrometry [59]. The physiological NO release from heart mitochondria has been calculated as 1.8 nmol NO × min^{-1} × mg $protein^{-1}$, considering the simultaneous oxidation of 3 mol of NADH (malate-glutamate) and 1 mol of succinate within the matrix, and taking into account that heart mitochondria oscillate between a NO-inhibited state 3 (47 %) and state 4 (53 %) [58]. In these conditions, NO release accounts for about 3 % of heart O_2 consumption.

Up to date, the immunochemical nature of heart mtNOS isoform is a subject under debate. The 51–57 % homology reported for nNOS, iNOS, and eNOS; and the cross-reactivity of isoform-specific anti-NOS antibodies, as well as the possible overexpression of iNOS and eNOS in experimental models could provide an explanation for the conflicting results [60]. However, the most convincing data implicate nNOS as the primary candidate for the NOS isoenzyme targeted into mitochondria [57, 60, 61]. In 2002, the sequence of rat liver mtNOS was reported as the α splice variant of the nNOS isoform, with post-translational modifications: acylation with myristic acid at the N-terminal and phosphorylation at the C-terminal region [61]. In the study of Kanai et al. [57], the NO production of an individual mouse cardiac mitochondrion was measured with a microsensor placed at the cytoplasmic face of the mitochondrial outer membrane. The sensor detected a NO signal in mitochondria isolated from eNOS-/- or iNOS-/- animals, while no signal was detected in mitochondria from nNOS-/- mice. In our laboratory, we have detected an increase in heart mtNOS expression after the exposure of rats to high altitude, using anti-nNOS and anti-iNOS antibodies [51]. Moreover, a decrease in heart mtNOS expression during the regression of the cardioprotection conferred by hypoxia was reported using anti-nNOS and anti-iNOS [50].

5 Regulation of Heart Mitochondrial NOS

Several situations can regulate heart mtNOS activity or expression. As an example, treatment with enalapril increased the production of NO by heart mitochondria [39, 62]. Interestingly, a receptor for angiotensin was found in inner mitochondrial

membrane and it was suggested that the renin-angiotensin system directly regulates mitochondrial NO production [63]. We have reported that rats submitted to chronic hypoxia showed about 60 % enhanced heart mtNOS activity [47]. This up-regulation was associated with a preservation of heart contractility upon aging or after an ischemic insult. Concordant results were observed when rats were exposed to natural high altitude [46, 51]. Again, heart mtNOS activity and expression were specifically increased after exposure [51] and this enhancement showed a similar pattern to the one observed for hematocrit. Another study performed by us showed a decline of about 30 % in mitochondrial NO production after ischemia-reperfusion, without modification of mtNOS expression, together with a decrease of 30 % in mitochondrial complex I activity [19]. This latter mitochondrial dysfunction was named by Boveris et al. "complex I syndrome" in which complex I and mtNOS are partially inactivated associated with protein nitration and oxidative damage to proteins and phospholipids [64]. Localization of NO production within mitochondria provides a reciprocal regulation between mtNOS and the intramitochondrial medium (Fig. 4.2). Indeed, heart mtNOS activity depends on intramitochondrial Ca^{2+} concentration [40, 65]; the blockade of mitochondrial Ca^{2+} uniporter inhibits mitochondrial NO production. In energized mitochondria, elevation of extramitochondrial Ca^{2+} stimulates mtNOS activity and decreases respiration. Interestingly, results from our laboratory showed that energized and coupled heart mitochondria produce NO without supplementation with Ca^{2+} [66], indicating that the concentration of Ca^{2+} in the preparation may be sufficient to sustain a basal mtNOS activity. Concerning the

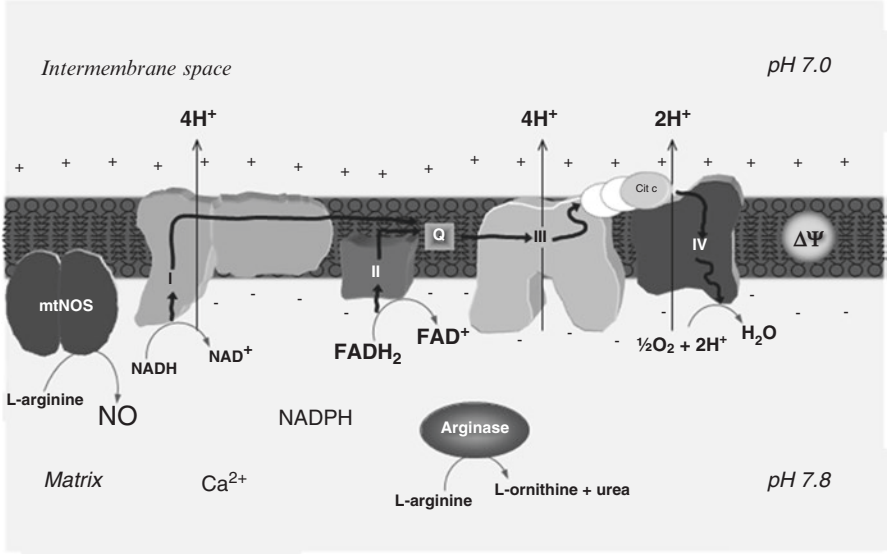

Fig. 4.2 Diagram showing the location of mtNOS within mitochondrion. Specific regulation of mitochondrial NO production occurs by the intramitochondrial levels of Ca^{2+}, O_2, L-arginine, arginases, and NADPH and by mitochondrial metabolic state and $\Delta\psi$

mtNOS activity determined in heart mitochondrial membranes, NO production was 80 % lower in the absence of Ca^{2+} in the reaction medium. Taking into account that heart mitochondrial matrix and cytosol Ca^{2+} concentrations are different [65–68], the existence of a Ca^{2+}-dependent NOS within mitochondria has significant consequences in terms of the differential regulation of this enzyme.

The mtNOS, as cytoplasmic NOS isoforms, requires O_2, L-arginine, and certain cofactors to produce NO. The study of NO production by heart mitochondrial membranes as a function of L-arginine concentration showed a hyperbolic response, with an apparent K_M value of about 35 μM [69]. Of note, rather lower intracellular levels of L-arginine and the presence of arginase-I and arginase-II activity in heart mitochondria have been reported [70]. The activity of mtNOS may be impaired under the conditions whereby L-arginine concentration within the heart mitochondria is diminished. As the content of NADP(H) in heart mitochondria is around 0.4 mM [71, 72], about 80 % of this nucleotide is in the reduced state (NADPH) and since NOS has a relatively low K_M value for NADPH (0.1–1 μM) [73], the intramitochondrial NADPH concentration is high enough to sustain mtNOS activity under physiological conditions.

The mitochondrial NO production is regulated by the metabolic state [49, 58, 66]. During the transition from resting (state 4) to active (state 3) respiration, heart mitochondrial NO release decreases about 60 %. The rate of NO release by mitochondria represents 10 % of the corresponding O_2 consumption in state 4 and only 1.5 % in state 3 [58]. This is consistent with the idea that mitochondrial respiration is more sensitive to exogenous NO in state 3 than in state 4 [12–15, 58, 66]. In addition, heart mitochondrial NO release shows an exponential dependence on the mitochondrial membrane potential ($\Delta\psi$). This dependence is more pronounced in the physiological range of $\Delta\psi$ (150 to 180 mV), where small changes in the $\Delta\psi$ produce noticeable variations of mitochondrial NO release [58, 60]. To date, several studies have shown that abolishing $\Delta\psi$ inhibits NO production by mtNOS activity, indicating a tight regulatory interplay between mitochondrial $\Delta\psi$ and NO production. Moreover, data from our laboratory have showed that heart inside out submitochondrial particles produce NO supported by succinate-dependent reversed electron flow in the respiratory chain, indicating a functional association between mtNOS and complex I proteins [74].

6 Conclusions

Nitric oxide is an essential molecule in the regulation of heart function in general, and of a range of key processes implicated in cardiac energy metabolism in particular. The presence of mtNOS in the mitochondria of high-energy utilizing cells, such as cardiomyocytes, indicates a precise regulation of metabolic pathways in which these organelles are involved. Heart mtNOS allows NO to optimize the balance between cardiac energy production and utilization, and to regulate oxygen and nitrogen free radical productions and Ca^{2+} homeostasis. Furthermore, heart mtNOS is regulated by physiological, pathological, and pharmacological situations.

Moreover, a spatially restricted localization of NO within mitochondria permits the regulation of NOS activity by the local environment within individual organelles. Thus, this enzyme seems to be critical during the adaptation of heart mitochondria to changes in cellular bioenergetics.

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