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Strategic localization of heart mitochondrial NOS: a review of the evidence

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Zaobornyj T, Ghafourifar P. Strategic localization of heart mitochondrial NOS: a review of the evidence. *Am J Physiol Heart Circ Physiol* 303: H1283–H1293, 2012. First published September 28, 2012; doi:10.1152/ajpheart.00674.2011.—Heart mitochondria play a central role in cell energy provision and in signaling. Nitric oxide (NO) is a free radical with primary regulatory functions in the heart and involved in a broad array of key processes in cardiac metabolism. Specific NO synthase (NOS) isoforms are confined to distinct locations in cardiomyocytes. The present article reviews the chemical reactions through which NO interacts with biomolecules and exerts some of its crucial roles. Specifically, the article discusses the reactions of NO with mitochondrial targets and the subcellular localization of NOS within the myocardium and analyzes the available data about heart mitochondrial NOS activity and identity. The article also describes the regulation of heart mtNOS by the distinctive mitochondrial environment by showing the effects of Ca^{2+}, O_2, L-arginine, mitochondrial transmembrane potential, and the metabolic states on heart mitochondrial NO production. The article depicts the effects of NO on heart function and highlights the relevance of NO production within mitochondria. Finally, the evidence on the functional implications of heart mitochondrial NOS is delineated with emphasis on chronic hypoxia and ischemia-reperfusion studies.

mitochondrial nitric oxide synthase; calcium; transmembrane potential; hypoxia; ischemia-reperfusion

SINCE THE RECOGNITION OF MITOCHONDRIA as the powerhouses of the cells, numerous studies have shown that these organelles play a central role in various biological processes. Under physiological conditions, mitochondria provide the cell with both the energy and the signals involved in the genetic expression and metabolic regulation (35). About two decades ago, the discovery that nitric oxide (NO) is the endothelium-derived relaxing factor caused a paradigm shift in the understanding of cardiovascular physiology and pathophysiology (68, 102). NO is a gaseous uncharged 30-Da molecule. It is highly diffusible in aqueous and lipid phases and possesses a biochemistry that supports its role as one of the most versatile molecules in various biological regulatory and signaling processes (76, 86, 134).

In the cardiovascular system, NO plays integral roles not only in the regulation of vascular smooth muscle tone but also in the function of ion channels, myocyte contraction, O_2 consumption, apoptosis, and hypertrophic myocardial remodeling (36, 92). In cardiac endothelial cells and myocytes, NO is generated by NO synthase (NOS) isozymes: neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2), and endothelial NOS (eNOS or NOS3) (71, 75). Initially, eNOS was considered to be the only isoform constitutively expressed in myocytes and thus the source of NO involved in the autocrine regulation of myocardial contraction and Ca^{2+} homeostasis (7, 37). However, subsequent studies showed that NOS is targeted to the cardiac sarcoplasmic reticulum (SR) (137) and localized in cardiac mitochondria (72). Additionally, iNOS has been shown to be expressed in the myocardium upon induction by cytokines (8) during inflammatory responses implicated in many pathophysiological conditions. There is growing evidence supporting the initial reports on the existence of a mitochondrial NOS (mtNOS) (54, 58) and the involvement of mitochondrial NO in the regulation of cellular functions. Nevertheless, some studies failed to show heart mitochondrial NO production, and the evidence about lack of existence of mtNOS within the heart and the liver has been published by these authors (21, 32, 82, 124). Interestingly, the detection limitation of the assays used in these studies has been reviewed and alternative methods have been suggested (53).

Although NO is highly diffusible, signaling functions of this molecule produced by specific NOS isoforms seem to be compartmentalized in distinct cellular microdomains and to modulate cardiac function differently. This article reviews the evidence about the NOS activity in heart mitochondria and about the impact of mtNOS in cardiac function under physiological and pathological conditions.

Biochemistry of NO in the Heart

The effects of NO in the heart stem from its production within different cellular compartments and its interactions with several biomolecules involved in distinct downstream signaling pathways. It is well known that NO activates soluble guanylate cyclase by binding reversibly to the heme iron of soluble guanylate cyclase and by forming a Fe-nitrosyl complex with the enzyme (134). This activation leads to the

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production of 3′,5′-cyclic guanosine monophosphate (cGMP) that stimulates protein kinase G and cGMP-regulated phosphodiesterases activities (83).

Certain key functions of NO in signaling and regulation of cardiac function are performed through cGMP-independent pathways including those that involve mitochondria (104). At physiological submicromolar concentrations, most distinct effects of NO on mitochondria are exerted on the respiratory chain. First, NO competes with O_2 for the binding site at the binuclear center of cytochrome *c* oxidoreductase. This leads to a high affinity and reversible inhibition of cytochrome *c* oxidase activity (24, 26). A second important effect of NO on mitochondrial respiratory chain is accomplished through the reaction of NO with the respiratory complex III, ubiquinol-cytochrome *c* oxidase. The exact mechanism underlying this effect in not fully understood. However, it has been shown that interaction of NO with mitochondrial respiratory complex III inhibits the electron transfer and enhances the production of superoxide anion (O_2^-) (108, 109, 127). In the matrix, the manganese superoxide dismutase catalyzes the reaction of two molecules of O_2^- to form hydrogen peroxide (H_2O_2), an uncharged species involved in the control of cell proliferation and death (51).

Other effects of NO in cell signaling are conveyed via nitrosation of proteins (121, 123). In nitrosation reactions, NO reacts reversibly with the nucleophilic centers in thiol residues contained within a specific consensus sequence of amino acids (123, 135) of a broad array of low molecular weight compounds (117) or proteins (120). When mitochondria are treated with NO donors, complex I is *S*-nitrosated resulting in significant inhibition of this respiratory complex (14, 25).

NO also reacts with O_2^- that is formed by the mitochondrial respiratory chain during normal or pathological oxygen metabolism. This reaction occurs at a rate constant of about 2×10^{10} $M^{-1} \cdot s^{-1}$, and its velocity is controlled by the diffusion rate of the reactants (77). The product is peroxynitrite ($ONOO^-$), a nonradical species capable of oxidizing and nitrating biomolecules and damaging irreversibly those targets (112). This NO congener may hinder mitochondrial functions and cause cell death. $ONOO^-$ also influences cardiac contractility and, in some cases, produces effects that are markedly different from those of NO (74, 93). Diverse factors participate in the switch from reversible inhibition of cellular respiration by NO to the pathological inhibition of mitochondrial function by $ONOO^-$. Distinct features of mitochondria make the organelles particularly important targets for biological effects of NO.

Heart NOSs

In the heart, specific NOS isoforms are distinctly located in cardiomyocytes (63, 144), and such compartmentalization provides a unique condition whereby the enzyme produces NO nearby target effectors within the same subcellular region (Fig. 1). While NO is highly diffusible, the distances that this free radical effectively travels are short due to the high reactivity of NO with several species abundantly available in most biological environments. In mitochondria, these species include heme irons, O_2^-, and thiols. Thus the different cardiac NOSs localized at specific parts of the myocytes allow the NO produced within these parts to interact coordinately and restrictedly with the colocalized effectors.

In cardiac myocytes, eNOS is localized at the caveolae of the sarcolemma and T tubules, where it is associated with the scaffolding protein caveolin-3, the myocyte-specific structural protein of caveolae (46, 64). The colocalization of eNOS with β-adrenergic receptors and Ca^{2+} channels allows eNOS-gen-

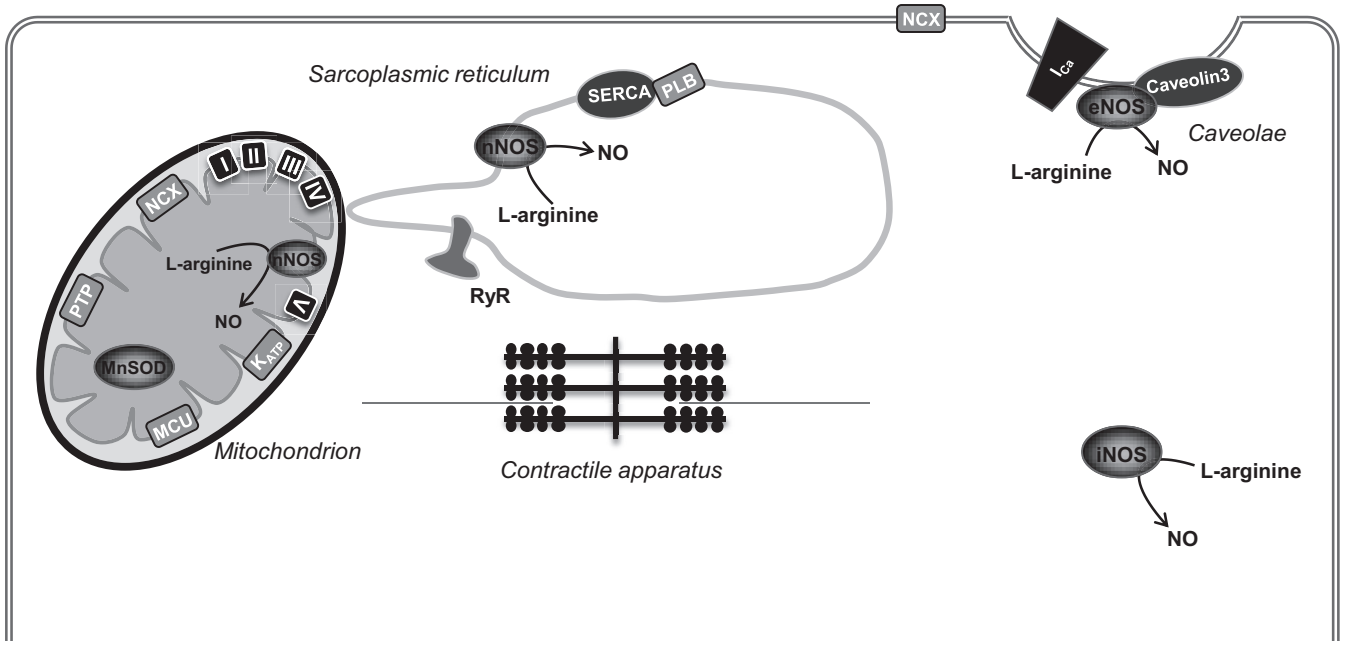

Fig. 1. Spatial localization of nitric oxide (NO) synthase (NOS) within cardiomyocytes. Mitochondrial NOS (mtNOS) is found close to the respiratory chain, endothelial NOS (eNOS) is enriched in caveolar and T-tubular structures close to the diads, neuronal NOS (nNOS) is located at sarcoplasmic reticulum membranes close to Ca^{2+} channels, and inducible NOS (iNOS) is expressed in the cytosol during pathophysiological conditions. NCX, Na^+/Ca^{2+} exchanger; PTP, permeability transition pore; MCU, mitochondrial Ca^{2+} uniporter; MnSOD, manganese superoxide dismutase; K_{ATP}, ATP-dependent K^+ channel; SERCA, sarco(endo)plasmic reticulum Ca^{2+}-ATPase; RyR, ryanodine receptor; I_{Ca}, Ca^{2+} current; I–V, complexes I–V, respectively.

erated NO to remain within diffusion distance of its molecular targets (9). Expression of eNOS in the myocardium is not limited to the cardiac myocytes. This NOS isoform is also expressed in endothelial cells (103) where NO exerts a pivotal role in the regulation of vascular tone. nNOS is localized at the SR, and under physiological conditions, it coimmunoprecipitates with ryanodine receptor. It also contributes to the regulation of myocardial function mainly by influencing myocyte Ca^{2+} handling (9, 137). nNOS-generated NO readily activates ryanodine receptor to release Ca^{2+} from the SR. Such Ca^{2+} release causes a positive inotropic effect, thus mediating an action on myocardial contractile function, i.e., the positive inotropism that seems to be opposite to that of eNOS (9). iNOS, although not constitutively expressed in the myocardium, has been detected in heart tissues from patients with heart failure (37, 65). There is a general consensus that iNOS is transiently expressed in the heart during immune responses under stress (8) and, therefore, during pathophysiological conditions of the myocardium such as in ischemia-reperfusion injury (133), septicemia (125), heart failure (146), as well as during aging (138).

During the last fifteen years, different groups have used various experimental approaches and reported the presence of mtNOS in the heart (1, 10, 16, 40, 45, 60, 66, 72, 79, 91, 115, 129, 130, 140, 141, 143, 147). Regarding the localization of mtNOS, one interesting study provided evidence about the docking of eNOS to the outer mitochondrial membrane in endothelial cells (52). Mutant eNOS lacking a pentabasic amino acid sequence within the autoinhibitory domain showed dramatically reduced binding to the mitochondrial membrane, which was associated with increased oxygen consumption. On the other hand, a recent study showed the presence of angiotensin II type-2 receptors in inner mitochondrial membrane. They detected a NO response in isolated mitochondria associated to impaired O_2 consumption (1). Strategic localization of mtNOS in organelles that are in charge of cellular energy metabolism in high energy-utilizing cells such as cardiomyocytes indicates a pivotal control of processes whereby mitochondria play essential roles. Moreover, a spatially restricted intracellular localization of NO within mitochondria permits regulation of NOS activity by the local environment within individual organelles. Such reciprocal interaction between heart mitochondria and heart mtNOS might provide a unique distinction between mtNOS and non-mitochondrial NOS isoforms. Nevertheless, conflicting results have appeared in the literature during the last years, and the current view about heart mtNOS identity and relevance is incomplete.

Mitochondrial NOS. An immunohistochemical study used silver immunogold labeling method to stain heart, skeletal muscle, and kidney mitochondria and showed that about 85% of the heart mitochondria were positive for the eNOS label (10). This report postulated that eNOS located in mitochondria could exert a ubiquitous regulation of oxidative phosphorylation. Later, two groups almost simultaneously proved the existence of a functional mtNOS in rat liver mitochondria localized at the inner mitochondrial membrane (54, 58). Since then, the presence of NO in the mitochondria has been demonstrated by using several methods. Confocal microscopy using fluorescent probes for coupled mitochondria (MitoTracker) and NO (4,5-diaminofluorescein diacetate) indicated the presence of NO in mitochondria (87). Also, direct evidence of mitochondrial NO production in endothelial cells was provided with similar experimental approaches (20, 38, 41). Technical limitations of the methods used to detect NO in biological environments, such as the lack of specificity of fluorescent probes, include those used for detection of NO in mitochondria. Other studies have detected heart mitochondrial NO release using a direct spin trapping technique with electron paramagnetic resonance spectrometry (58, 66). Additionally, NO concentration within mitochondria, which results from its production and consumption by mitochondria, has been estimated to be in the nanomolar range (17). Heart mtNOS has been studied by functional experiments in which mitochondrial membrane potential-dependent NO production was measured in imaging experiments following permeabilization of cardiomyocytes (40) (Fig. 2*A*) or directly detected from isolated cardiac mitochondria (72, 143) (Fig. 2*C*). Studies of cardiac mtNOS have used various methods including immunohistochemistry (10, 66), spectrophotometry (16, 30, 45, 79, 115, 141, 142), radiometry (139, 143), fluorometry (40, 139, 143, 147), chemiluminescence (143), and electrochemistry (72). On the other hand, one study reported that the ubiquinone/cyt bc_1 is a site where nitrite is reduced to form NO congeners within mitochondria in a NOS-independent manner (100). Despite the conflicting evidence about mtNOS (21, 81), some authors concur on that heart mtNOS is relevant in physiological and pathological functions of heart mitochondria, with implications in ischemia-reperfusion injury and right ventricle hypertrophy (67, 96).

The short mitochondrial genome encodes for only few proteins, none of which resembles cytoplasmic NOS. Therefore, if mtNOS is one of the cytoplasmic NOS isoforms, one of the nuclear-encoded NOS isoforms should be transported to the mitochondria after the protein is synthesized in the cytosol. All three NOS isoforms have been proposed to be located within mitochondria of different cells. In the heart, however, the most convincing data implicate nNOS as the primary candidate for the cytoplasmic NOS izosyme targeted into mitochondria (40, 72, 142). Evidence in favor of eNOS (10, 66, 140) and iNOS (45, 60, 79, 129) (Fig. 2*B*) presence in heart mitochondria has also been published. One study purified and analyzed mtNOS from rat liver by using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF). The sequence of the purified protein was reported as the α-splice variant of the nNOS with acylation with myristic acid at the NH_2-terminal and phosphorylation at the COOH-terminal region (44). This report was not confirmed by others by using seven different NOS antibodies, and it was concluded that NOS is absent in mitochondria (81). Another study showed a NOS activity in mouse cardiac mitochondria by measuring the NO production of an individual mitochondrion with a porphyrinic microsensor placed at the cytoplasmic face of the mitochondrial outer membrane. The sensor detected NO upon addition of Ca^{2+} to the reaction medium containing mitochondria isolated from control mice. The NO signal was detected for mitochondria isolated from $eNOS^{-/-}$ or $iNOS^{-/-}$ animals, whereas no signal was detected in mitochondria from $nNOS^{-/-}$ mice (72) (Fig. 2*C*). From nNOS alternative-splice variants currently described (111), only the nNOSα is knocked out in $nNOS^{-/-}$ mice by deleting the exon 2 near the NH_2-terminus. Nevertheless, the exact nature of heart mtNOS isoform is still not elucidated, a situation that extends to other reported mtNOS activities (10, 50,

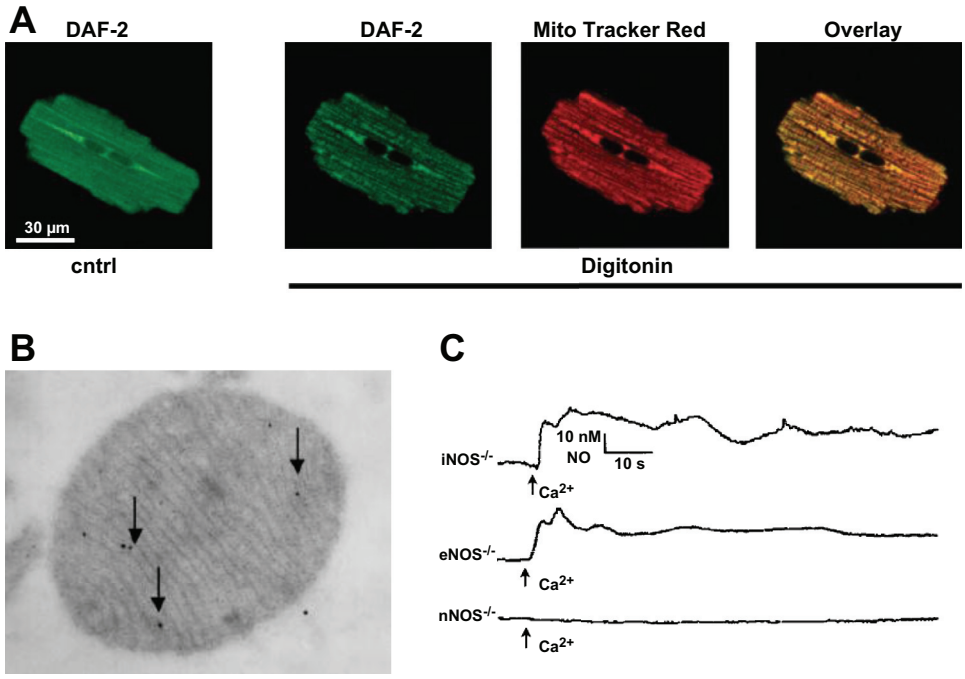

Fig. 2. Heart mtNOS. *A*: measurements of NO production by mitochondria of digitonin-permeabilized ventricular myocytes. *A*, *left* to *right*: confocal images of a ventricular myocyte loaded with 4,5-diaminofluorescein (DAF-2; green) and MitoTracker Red (red) before [control (cntrl)] and after cell permeabilization with digitonin. *A*, *right*: overlay of the 2 individual images. Colocalization of DAF-2 and MitoTracker Red is represented by shades of yellow (adapted with permission from Ref. 40). *B*: representative immunoelectron micrographs of isolated mitochondria from rat heart using an anti-iNOS antibody. Arrows indicate 10-nm gold particles on inner mitochondrial membranes. (Adapted with permission from Ref. 45). *C*: identification of the isoform of cardiac mtNOS was deduced by using mitochondria isolated from the hearts of knockout mice for the $nNOS^{-/-}$, $iNOS^{-/-}$, and $eNOS^{-/-}$ isoforms. Only the mitochondria isolated from the hearts of $nNOS^{-/-}$ mice failed to produce NO (adapted with permission from Ref. 72).

66, 81). The 51 to 57% homology reported for nNOS, iNOS, and eNOS and the cross-reactivity of isoform-specific anti-NOS antibodies could provide an explanation for the incompatible results (73). Moreover, mitochondrial preparations were shown to contain numerous proteins across a wide range of molecular weights that can react with anti-NOS antibodies (21).

Regulation of NO production within mitochondria. Figure 3 shows that localization of NO production within mitochondria provides a distinct specific reciprocal regulation between mtNOS and intramitochondrial Ca^{2+}, pH, L-arginine, O_2, or redox state (36). Indeed, heart mtNOS activity is dependent on cation concentration in the reaction medium. Extramitochondrial free Mg^{2+}, a well-known mitochondrial Ca^{2+} uniporter blocker, inhibits NO production in rat heart mitochondria by 60% in a dose-dependent manner (91, 143). This concept is in agreement with other reports (40, 41) showing that blocking the mitochondrial Ca^{2+} uniporter with ruthenium red inhibits mitochondrial NO production. In intact energized mitochondria, elevation of extramitochondrial Ca^{2+} is sufficient to stimulate mtNOS activity and to decrease the respiration rate. Interestingly, substrate-energized heart mitochondria produce NO without additional supplementation with Ca^{2+} (128), indicating that the concentration of Ca^{2+} in the mitochondrial preparation may be sufficient to sustain a basal mtNOS activity.

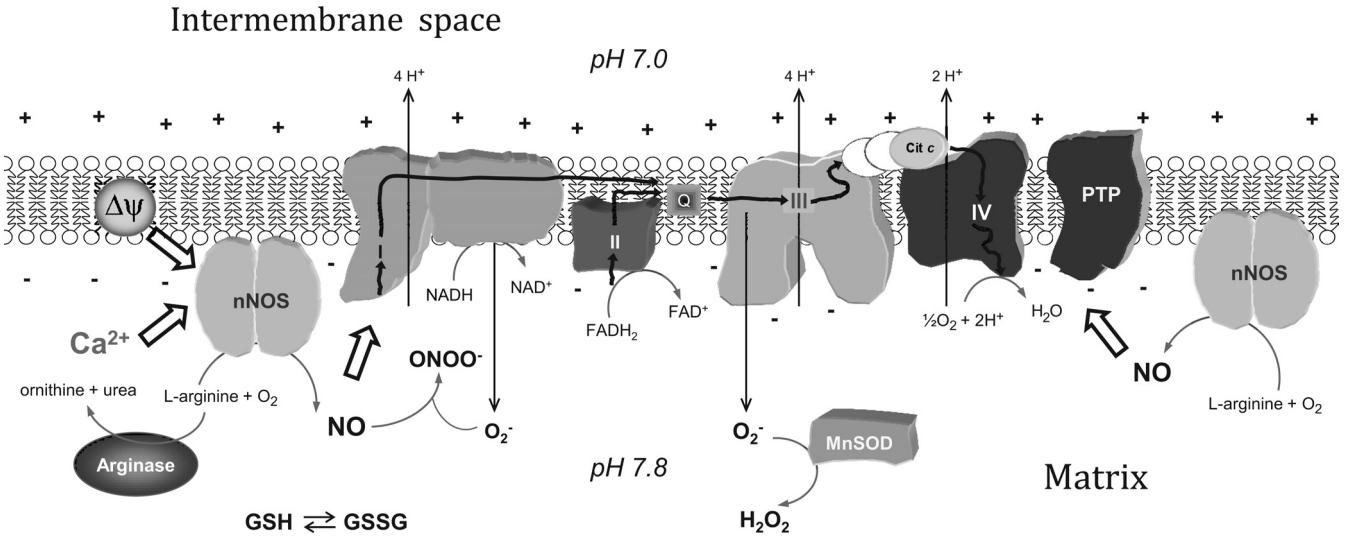

Fig. 3. Reciprocal regulation of mtNOS activity and mitochondrial function. Localization of NOS within mitochondria provides a distinct specific regulation of mitochondrial NO production by intramitochondrial O_2, Ca^{2+}, pH, mitochondrial transmembrane potential ($\Delta\psi$), L-arginine, arginases, or redox state (GSH/GSGG balance). NO produced by mtNOS can readily react with mitochondrial targets such as respiratory complexes I, III, or IV or mitochondrial PTP. The superoxide anion (O_2^-) is formed at the respiratory chain and undergoes a very fast reaction with NO to form peroxynitrite ($ONOO^-$), or it is catabolized by MnSOD to form H_2O_2. Cit *c*, cytochrome *c*.

Nevertheless, the functional studies performed on cardiac mitochondria isolated from mice (70, 72) or rats (91, 115, 126, 140, 141, 143) demonstrated the activation of mitochondrial NO production upon addition of Ca^{2+} to the reaction medium. Taking into account that heart mitochondrial matrix Ca^{2+} concentrations differ from cytosolic Ca^{2+} concentrations (39, 85), the existence of a Ca^{2+}-dependent NOS within mitochondria has significant consequences in terms of the differential regulation of this enzyme.

Mitochondrial NO production is influenced by mitochondrial metabolic state (17, 126, 128). During the transition from resting (state 4) to active (state 3) respiration, heart mitochondrial NO release decreases markedly (about 60%), from 2.2. to 1.2 nmol NO/min·mg protein (126). Interestingly, the rate of NO release by isolated heart mitochondria at state 4 represents about 10% of the corresponding O_2 consumption, whereas at state 3 the rate of NO release represents only about 1.5% of O_2 uptake (17, 128). This is consistent with the hypothesis that mitochondrial respiration at physiological O_2 levels is more sensitive to exogenous NO in state 3 than in state 4 (23). To note, the fact that intact mitochondria produce NO at an estimated rate of about 1.2–2.2 nmol NO/min·mg protein leads to the notion that the NO concentration resulting from this production should be in the micromolar range. However, the estimated steady-state concentration of NO within mitochondria has been reported to remain in the nanomolar range (17). This latter datum indicates that a substantial fraction of the NO produced in mitochondria reacts with mitochondrial targets such as O_2^-, heme groups, and thiols. In addition, heart mitochondrial NO release shows a strong and exponential dependence on the mitochondrial transmembrane potential ($\Delta\psi$) (17, 128). This dependence is more pronounced in the physiological range of $\Delta\psi$, that is between 150 and 180 mV, where small changes in the $\Delta\psi$ cause noticeable variations in mitochondrial NO release. In addition, a marked decrease of mtNOS activity has been observed when $\Delta\psi$ was collapsed by an uncoupler. To date, several studies have shown that abolishing $\Delta\psi$ inhibits mtNOS activity, indicating a tight regulatory interplay between mitochondrial NO production and $\Delta\psi$ (41, 72, 143).

The mtNOS, same as cytoplasmic NOS isoforms, requires O_2, L-arginine, and certain cofactors to produce NO. The apparent O_2 K_m for heart mtNOS is 40 μM (3), suggesting that under physiological conditions whereby O_2 concentration is in the 5–20 μM range (28, 59) the mtNOS activity is oxygen limited. In this way, the in vivo enzymatic activity of heart mtNOS might be 6–25% of its maximal activity (18).

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, a concentration that is well within the estimated liver mitochondrial L-arginine levels of 150–300 μM (3, 128). However, rather lower intracellular levels of L-arginine were reported in cardiomyocytes (42). Moreover, the expression of arginase II in mitochondria of mouse cardiac myocytes (122) and arginase I in feline cardiac myocytes (69) has been demonstrated. The activity of mtNOS may be impaired under the conditions whereby L-arginine concentration within the heart mitochondria is diminished.

Role of NO in the Regulation of Heart Function

The influence of NO on myocardium varies greatly depending on determinants including the NOS isoform activated, the rate of NO production, the signaling pathway triggered, and the redox environment within the site of NO production. Therefore, it is not surprising that paradoxical results exist about both positive and negative effects of NO and its related congeners in the heart (145). As described above, the specificity of NO regulation in the heart is conferred through spatial localization of NOSs to distinct signaling microdomains (9). Thus eNOS is enriched in caveolar and T-tubular structures close to the diads, nNOS is found at SR membranes close to ion channels, and mtNOS is located in mitochondria close to the energy transduction and O_2^- production machinery. NO influences myocardial contractility, ventricular relaxation, mitochondrial respiration, and energy production through the distinct mechanisms discussed earlier in this review, and its concentration fluctuates within the cardiac cycle (107).

The presence of NOS in cardiac mitochondria provides a mechanism of fine regulation of the respiratory complexes and enzymes of the citric acid cycle (Fig. 3). Many studies indicate that mitochondrial NO regulates energy metabolism (94, 109) and inhibits cytochrome oxidase (4, 24, 26). In studies with muscle and isolated beating heart, NO donors and agonists such as bradykinin suppress tissue O_2 uptake and H_2O_2 production (136). Moreover, it has been reported that NO increases oxidative phosphorylation efficiency (27) and regulates mitochondrial matrix pH, $\Delta\psi$, and Ca^{2+} buffering capacity (55). Thus NO not only regulates muscular contraction that is the major energy consuming process of the heart but also diminishes mitochondrial energy production (116). In addition, mitochondria are major sources of reactive oxygen species such as O_2^- and H_2O_2 (15). Through its interaction with components of the electron transport chain, NO functions not only as a physiological inhibitor of cell respiration but also enhances the generation of reactive species (108) derived from the incomplete reduction of O_2 by mitochondria and thereby triggers various mechanisms underlying the survival or death of the cells (94).

Functional relevance of mtNOS in heart physiology and pathology. The relevance of mtNOS in mitochondrial bioenergetics and cell physiology has been the subject of many studies. Pivotal roles of mtNOS have been postulated in chronic hypoxia, ischemia-reperfusion, cell signaling, aging, dystrophin deficiency, inflammation, and cancer. The mtNOS activity has been found upregulated in cold acclimation (105). Treatment with enalapril, an angiotensin-converting enzyme inhibitor, produced an increase in the production of NO by heart mitochondrial membranes (16, 30). Lately, a receptor for angiotensin was found located in inner mitochondrial membrane, and it was suggested that the renin-angiotensin system directly regulates mitochondrial NO production (1). In addition, heart mtNOS activity was increased in experimental endotoxemia after LPS treatment (3). Therefore, a variety of physiological, pathological, and pharmacological situations can cause changes in heart mtNOS activity or expression.

Figure 3 depicts distinct effects of NO on mitochondria, such as competitive inhibition of respiration (4, 24, 26, 108, 109), regulation of the rate of production of species derived from O_2 partial reduction (108), as well as prevention or

activation of mitochondrial permeability transition pore (mPTP) (31, 40, 84, 131), and participation in Ca^{2+} homeostasis (40). Interestingly, the reversible inhibition of O_2 consumption shows a dependence on both O_2 and NO concentrations, competition between O_2 and NO, and different effects of NO in different mitochondrial metabolic states (4, 5). Additionally, NO inhibits the electron transfer chain in the cytochromes *b-c* segment in complex III (108, 109) by possibly binding to iron-sulfur centers that are intermediates of electron transfers. This reaction increases the level of the semiquinone of ubiquinol that upon autoxidation reduces O_2 to O_2^-. Moreover, complex I is a target for reversible inhibition by *S*-nitrosation of critical thiol residues, because its inhibition by NO can be reversed by light or reagents containing thiol groups (14). These findings are in accordance with the structural and functional interaction observed among complexes I and IV and mtNOS (49, 106). A physical proximity of mtNOS with the COOH-terminal peptide of the Va subunit of cytochrome *c* oxidase was shown by electron microscopic immunolocalization and coimmunoprecipitation studies. Other report showed that not only complex IV but also complex I proteins immunoprecipitate with intramitochondrial and translocated nNOS, which indicates a direct molecular interaction between mtNOS and complexes I and IV (49, 106).

The ability of mtNOS to regulate mitochondrial O_2 uptake and H_2O_2 production through the interaction of NO with the respiratory chain has been named mtNOS functional activity (16, 127). This functional activity is determined by the difference in the rates of O_2 uptake or H_2O_2 production in isolated mitochondria in two conditions. Under the condition when NO steady-state levels are the highest, such as in the presence of sufficient L-arginine and SOD, mitochondrial active O_2 consumption is impaired and mitochondrial H_2O_2 production is enhanced. On the contrary, when NO steady-state levels are the lowest, mitochondrial active O_2 consumption is increased and mitochondrial H_2O_2 production is decreased (127). Such situation is achieved in the absence of NOS substrates or cofactors or in the presence of a NOS inhibitor or a NOS scavenger, e.g., oxyhemoglobin. Changes in mtNOS functional activity reveal modifications of NO production by mtNOS. For instance, treatment with enalapril produces a marked increase in mitochondrial NO production associated with an enhancement in mtNOS functional activity, as measured through both the O_2 uptake and H_2O_2 production (16, 127).

Some reports have established important functional implications of mtNOS activity (19, 40, 55, 56). Those studies have shown that loading mitochondria with Ca^{2+} decreases matrix pH in a N^{ω}-monomethyl-L-arginine-sensitive manner (2). This acidification was related to a decrease in the proton extrusion from the matrix to the intermembrane space and a drop in the $\Delta\psi$, a consequence of the inhibition of the respiratory chain. Moreover, it has been suggested that Ca^{2+} uptake by respiring mitochondria stimulates mtNOS, elevates $ONOO^-$ formation, which in turn causes specific Ca^{2+} release from mitochondria followed by mtNOS inactivation (2). Evidence for $ONOO^-$ formation by mtNOS was originally observed in rat liver mitochondria (19, 56). It was demonstrated that mtNOS-mediated $ONOO^-$ formation promoted Ca^{2+} (19) and cytochrome *c* (56, 57) release from mitochondria, leading to a Bcl-2-dependent apoptosis. Moreover, exogenous and endogenously produced NO reduces mitochondrial Ca^{2+} accumulation (38), providing a means by which mtNOS can influence mitochondrial metabolism as well as survival. In this way, NO generated within mitochondria regulates matrix pH, $\Delta\psi$, and Ca^{2+} buffering capacity. Such a wide spectrum of actions indicates that the interaction of NO and mitochondria plays critical roles in heart homeostasis.

HEART CHRONIC HYPOXIA. Adaptation to chronic hypoxia has been shown to confer the heart an improved tolerance to all major deleterious consequences of acute O_2 deprivation, resulting in cardioprotection, in a similar way to preconditioning (98). NO has been extensively proposed as one of the molecular messengers involved in this type of adaptation (6). However, the subcellular localization and the identity of the NOS implicated in the increased tolerance to ischemic injury remains to be fully established. Different groups have reported iNOS upregulation during the exposition of rats to chronic hypoxia (62, 114). Some reports indicate that eNOS is upregulated during rabbit (47) and rat (48) heart adaptation.

Studies performed on mitochondrial fractions indicate that chronic hypoxia upregulates mtNOS. Rats submitted to simulated chronic hypoxia using a hypobaric chamber showed right ventricle hypertrophy, increased hematocrit values (78), and about 60% enhanced activity of heart mtNOS (141). This upregulation was associated with a preservation of the papillary muscle contractile parameters upon aging and improved tolerance to postischemic contractile dysfunction. Using a similar approach, a decrease in heart mtNOS activity and expression during the regression of the cardioprotection conferred by hypoxia has been reported (79). Other studies showed that liver mtNOS activity is upregulated after acute exposure to hypoxia (80, 118).

These data are in agreement with the results obtained using a natural high altitude model, where rat heart mtNOS activity and expression were increased after 42–84 days of exposure (60, 142). Rats submitted to high altitude also showed right ventricle hypertrophy. The enhancement in heart mtNOS activity showed a pattern similar to the one observed for hematocrit (142). The effect was selective for heart mtNOS because the activities of liver mtNOS, nonmitochondrial heart NOS, and heart mitochondrial complexes were unchanged (60).

These models of heart mtNOS upregulation in chronic hypoxia are relevant to chronic myocardial hypoxia involved in human coronary diseases. The increase in mitochondrial NO release, which decreases the consumption of O_2 by the respiratory chain, allows O_2 to diffuse further and to reach more mitochondria (86, 109), providing a way of increasing O_2 extraction by the tissues. There is an increased requirement for energy during chronic exposure to hypoxia when O_2 availability is low. Additional changes in cardiomyocyte to satisfy the energy demands of the cell include an increase in the number of mitochondria, more homogenous distribution of the organelles, and a decrease in the mitochondrial size (29, 101). NO triggers mitochondrial biogenesis in several cell types and tissues, including those obtained from the heart, through a cGMP-dependent mechanism (99). The enhanced mitochondrial generation of reactive oxygen species constitutes another event implicated in the onset of cardioprotection (11). One postulated mechanism whereby O_2^- and H_2O_2 may be distinguished as prominent mediators of adaptation to hypoxia involves the stabilization of transcription factor hypoxia-inducible factor-1α (11). Thus the NO produced by mtNOS may also

act at this stage of the sequence of events as a building block implicated in the construction of the cardioprotection established during adaptation to chronic hypoxia.

HEART ISCHEMIA/HYPOXIA AND REPERFUSION. Acute myocardial ischemia/hypoxia can result in myocyte dysfunction and death. Therefore, restoration of the blood flow seems to be essential to prevent cell damage. However, the reoxygenation of the hypoxic myocytes is one of the prime mechanisms underlying cell and tissue damage in pathological conditions including ischemic heart disease (61). A common feature of reperfusion is the increase in Ca^{2+} concentration. NO has been indicated as one of the key factors involved in the onset of cell damage in ischemia/hypoxia and reperfusion (13). Moreover, increased levels of oxidative species have been found under conditions associated with ischemia/hypoxia and reperfusion (25b). Pathological, i.e., excessive, levels of NO are likely to affect respiration by mechanisms qualitatively different from those observed during reversible physiological regulation. Increased $ONOO^-$, together with mitochondrial malfunctioning, is a hallmark of hypoxia-reperfusion injury in the heart (130). MtNOS-derived $ONOO^-$ causes oxidative modification of mitochondrial components and releases the key mitochondrial proapoptotic protein, cytochrome *c* (56, 97). Thus these organelles play a crucial role in oxidative injury during ischemia/hypoxia and reperfusion (113, 136).

An in vitro model that allows studying isolated mitochondria under hypoxia or hypoxia-reoxygenation showed that mitochondria, independent of other cellular components, respond to hypoxia-reoxygenation primarily by increasing the intramitochondrial Ca^{2+} levels through a shift in the balance between ionized and nonionized mitochondrial Ca^{2+} in favor of the ionized form (143). The mitochondrial production of NO is reduced during ischemia because NOS requires O_2 to generate NO and that low intracellular pH generated during ischemia inhibits the enzyme. During reperfusion, mtNOS produces a burst of NO, which results in the formation of $ONOO^-$. This latter damages proteins and suppresses O_2 consumption in the postischemic myocardium. Furthermore, elevated intramitochondrial ionized Ca^{2+} stimulates mtNOS and increases the mitochondrial production of O_2^- and $ONOO^-$, leading to oxidative and nitrative modifications of mitochondrial proteins, release of cytochrome *c* from mitochondria, oxidative modification of mitochondrial lipids and proteins, and inactivation of mitochondrial enzymes susceptible to $ONOO^-$ (143). In contrast, $ONOO^-$ has been reported as beneficial in a concentration-dependent manner in blood-perfused hearts exposed to hypoxia/reoxygenation (89).

At NO levels such as those reached during ischemia-reperfusion of cardiomyocytes, a reduction of $\Delta\psi$ is produced with the subsequent reduction of Ca^{2+} accumulation, thus protecting the cells from potentially lethal mitochondrial Ca^{2+} overload (88, 113). The cell injury and death observed after reoxygenation of the tissue are in accordance with several reports showing that NO can prevent or accelerate mPTP (31). This depends on whether NO concentrations are physiological or supraphysiological and whether O_2^- is generated simultaneously (40). While supraphysiological NO concentrations sensitize mPTP, physiological levels of NO inhibit mPTP opening with an IC_{50} of about 10 nM (22). The mPTP opens in response to high matrix Ca^{2+} concentrations, low ATP levels, and enhanced O_2^- production (90). Opening of the pore causes abrupt mitochondrial depolarization, ATP depletion, and necrotic or apoptotic cell death (12). In this regard, heart and liver mtNOS play important roles in mPTP regulation (40, 84, 147), suggesting relevance of mtNOS for yet another important mitochondrial function. On the other hand, the existence of alternative NOS-independent mechanisms of NO production that may operate in situations in which conventional NO production is impaired has been described (132). Inorganic nitrite (NO_2^-) can be chemically reduced back to NO in acidic media. During global ischemia, the pH in the heart falls to around 5.5, a condition that greatly accelerates reduction of NO_2^- to NO (148).

Finally, it has been suggested that a transient reversible inhibition of the mitochondrial electron transfer minimizes ischemia-reperfusion injury and that blockade of electron transport at complex I preserves respiration during reperfusion (95). Studies have shown that complex I inhibition by nitrosation protects mitochondria during hypoxia and reoxygenation and cardiomyocytes during ischemia-reperfusion (25a, 119). There is evidence that *S*-nitrosation of complex I proteins is associated with cytoprotection from the damage caused by ischemia-reperfusion (43, 110).

Conclusions and Perspectives

NO is a molecule of central importance for the regulation of heart function and involved in a broad array of key processes in cardiac metabolism. Different NOSs exist in different localizations within the cardiac cells and exert a particular influence in cardiac function. The present article reviewed some of the current knowledge about NO metabolism in the heart with an emphasis on heart mtNOS, summarizing both the evidence provided by the published studies and the controversies that still surround this enzyme. Certainly, further studies are required in this field for reaching a consensus. The regulation of mtNOS by $\Delta\psi$ and Ca^{2+} allows the enzyme to respond to changes in cardiomyocyte energy homeostasis. Moreover, alterations of mtNOS activity and expression during chronic hypoxia and ischemia-reperfusion support the relevance of this enzyme in long-term heart adaptation to decreased O_2 or in acute ischemic insult. At the same time, NO generated within mitochondria has focused effects on mitochondrial function. Together, heart mtNOS allows NO to optimize the balance between cardiac energy production and utilization and to regulate processes such as apoptosis, oxygen, and nitrogen free radical production and Ca^{2+} homeostasis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

T.Z. and P.G. conception and design of research; T.Z. prepared figures; T.Z. drafted manuscript; T.Z. and P.G. edited and revised manuscript; T.Z. and P.G. approved final version of manuscript.

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