[Frontiers in Bioscience 11, 2725-2738, September 1, 2006]

Critical overview of mitochondrial nitric-oxide synthase

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1. ABSTRACT

The recent discovery of mitochondrial nitric-oxide synthase (mtNOS) has provided novel information on mitochondrial biology. mtNOS, localized at the inner membrane, functions as one of the important regulatory factors that affect cellular respiration in mitochondria. The distribution of mtNOS in diverse organs of many species suggests its biological importance. Changes in mtNOS expression and activity in many pathophysiological situations may imply its significant involvement in various NO-related biological phenomena. The functional coordination of mtNOS with other NOSs present in the same or adjacent tissues is unknown as it is most of the regulatory mechanisms of mtNOS expression. Thus, future studies will be required to validate the physiological significance of mtNOS, and to elucidate its regulatory mechanisms on cellular energy metabolism in mitochondria.

2. INTRODUCTION

Nitric oxide (NO) is a short-lived gaseous free radical, which participates in diverse biochemical reactions. NO was first identified as an important endogenous signaling molecule (1-2). As a consequence, a significant numbers of studies have been published to explore its diverse biological functions. NO functions were identified originally in the vascular system. NO is part of the local vasodilatory effect (2), and of the inhibitory effect on platelet aggregation and adhesion (3). Besides these effects in the cardiovascular system, other important roles of NO have been reported in nervous and immune systems. For example, NO acts as a non-adrenergic, non-cholinergic neurotransmitter of corpus cavernosum of the penis (4), and gastrointestinal tract (5). Thus, the importance of NO in physiological and pathological situations is not limited to relaxing endothelium, but can be extended to other complex, important biological systems.

NO actions have been described as beneficial or harmful depending essentially on the concentration of NO produced and the localization of this production. At relatively low concentrations, NO mediates the anti-anginal and anti-ischaemic effects of nitroglycerine (6). Overproduction of NO leads to damage of the endothelium and smooth muscle cells during septic shock (7-8), or impairs neurons in certain neurodegenerative diseases (9). Furthermore, it has been indicated that NO could promote opposite effects such as tumor proliferation and tumor apoptosis (10).

Nitric oxide is produced by an enzyme named nitric-oxide synthase (NOS). This enzyme is classified into two categories according to the different expression forms: cNOS or constitutively expressed form, and iNOS or inducible form. In general, the constitutive forms produce low level of NO, mainly used as a signal molecule. iNOS has a higher rate of NO production and is activated in response to pathological changes like infection or inflammation (11). Constitutive NOSs require the activation by Ca^{2+}-calmodulin, whereas iNOS is less sensitive to calcium concentrations (12) because it is tightly bound to calmodulin. From the three NOS isoforms of which genes have been cloned, endothelial (eNOS) and neuronal (nNOS) are constitutive NOSs. However, isoform names do not necessarily reflect the tissue of origin, e.g., eNOS has been detected in cells other than endothelial (13). In this study, the isoforms will be referred by the following nomenclature according to current literature: nNOS (neuronal NOS, bNOS, NOS-1, and Type I), iNOS (inducible NOS, macNOS, mNOS, NOS-2 and Type II), eNOS (endothelial NOS, cNOS, NOS-3, and Type III).

Endogenous NO is synthesized by NOS using L-arginine and NADPH as a substrates, in the presence of oxygen. The enzyme contains a heme, and utilizes various cofactors such as tetrahydrobiopterin, FAD, calmodulin, calcium, and FMN (14). This reaction is catalyzed by all the three isoforms of NOS, resulting in the production of NO gas and L-citrulline. In detail, two successive reactions comprise this monooxygenation reaction: the first step is the conversion from L-arginine to Nw-hydroxy-L-arginine, an intermediate, with the use of one molecule of NADPH as an electron donor; the second step is the conversion from this intermediate to L-citrulline, with the use of half molecule of NADPH as an electron donor, resulting in the production of NO (12, 15).

Mitochondria are a key organelle for cellular energy metabolism; however, a link between mitochondria and NO had not been fully recognized until 1990s when immunohistochemical and biochemical studies presented evidence of NOS in these organelles (16-22). In the last decade, many novel facts on mtNOS have been elucidated although much of its physiological aspects have not yet been fully understood. The goal of this study is to present recent advances in mtNOS, illustrate issues not clarified in detail, and describe future research to be undertaken in this subject.

3. DISCOVERY OF mtNOS

The occurrence of NOS in rat skeletal muscle mitochondria was first suggested by a similar distribution immunohistochemical pattern of eNOS antigen and succinate dehydrogenase (16). In another study, a similar immunohistochemical pattern of nNOS and cytochrome oxidase was reported (19). Electron microscopy studies using gold immunolabelling of eNOS presented further indication of the occurrence of a NOS localized in mitochondria from rat liver, brain, heart, skeletal muscle, and kidney (17-18). Following these immunohistochemical studies, a breakthrough of mtNOS was brought by two independent laboratories, providing the first biochemical evidence of the NO production by mtNOS (20-22). Using mitochondria-rich fractions, Ghafourifar *et al* showed NOS activity in mitochondria, its modulation by calcium concentrations, and control of mitochondrial respiration by mtNOS (20). Using purified rat liver mitochondria (not enriched fractions which contain substantial contributions of other subcellular compartments) and two spectroscopic techniques, our laboratory provided the biochemical evidence of NO production by mtNOS and the unequivocal presence of this enzyme in mitochondria from hepatocytes, excluding macrophages or other cell contributions (21-22). Our lab provided the biochemical evidence for a regulation of the oxygen consumption at the cytochrome oxidase level, excluding other effects of NO on putative targets along the respiratory chain (21). After the occurrence of mtNOS was reported in rat liver, mtNOS has been suggested in brain (23-29), kidney (28-29), heart (30-32) and thymus (33-35). However, mitochondria-rich fractions from these organs are highly heterogeneous responding to the diverse tissue contribution. For example, “brain” mitochondria can be the result of neurons and glia, of synaptosomal and non-synaptosomal origin, among other options, of not purified accordingly.

4. ASSESSMENT OF mtNOS ACTIVITY

The evaluation of mtNOS activity has been proven to be experimentally challenging. Two factors may account for this issue; first, mtNOS activity cannot be detected when NO consumption in mitochondria exceeds NO production (36-37). Mitochondria are the organelle where a substantial amount of NO is consumed (38), and the main consumption location might be the inner mitochondrial membrane (39). Several pathways for NO consumption in mitochondria have been proposed; one of them may involve cytochrome *c* oxidase (40-43). When mtNOS activity is intended to be evaluated, controls such as cross-contamination with other subcellular compartments, hemoproteins (such as hemoglobin or myoglobin, released during the tissue or organelle isolation, which will bind effectively NO), arginase (which will consume arginine), coupling of the organelles, threshold and specificity of the analytical method that will be used to follow NO, should be accurately evaluated. In addition to these experimental issues, the rate of NO production by mtNOS is low (about 2 nmol/min mg protein) and the enzyme is not abundant when compared to other mitochondrial components.

Second, the short-life of NO as a gaseous free radical poses a problem due to its diffusion, hydrophobicity, and reactivity. Thus, every NO measurement method has limitations and pitfalls, and those should be accurately tested before reaching conclusions. Most of the researchers recruit a combination of methods to circumvent some of the above-indicated limitations.

4.1. Electron paramagnetic resonance (EPR)

EPR or electron-spin-resonance (ESR) spectroscopy is a technique that allows a chemical species with unpaired electrons like free radicals to be detected by measuring the absorption of microwave radiation (44-45). When the spin trap $(\text{monogalactosyl diacylglycerol})_2/Fe^{II}$ reacts with NO, a relatively stable NO-spin trap adduct is formed, and a unique triplet EPR spectrum can be observed. Several studies had used EPR to follow NO production using mitochondria (15, 21). Although this assay is very specific for NO, the detection level is rather low (micromolar levels). The stability of the NO-spin trap should be tested in each experimental setting for reduction of this adduct to EPR-silent species can be observed leading to false negatives.

4. 2. oxymyoglobin / oxyhemoglobin assays

NOS activity can be indirectly quantified by another spectrophotometric measurement, namely the oxidation of oxymyoglobin (oxyMb) to metmyoglobin (metMb) by NO (46-47). This reaction is considered to be one of the important processes for NO catabolism (48). The relative high sensitivity of this assay allows detecting nanomolar levels of NO. A relatively high concentration of Hb or Mb can be used to overcome the possible consumption of NO by other hemoproteins, thus favoring an accurate reflection of the rate of NO production. When using this method, it is highly recommended the use of a dual-wavelength, double-beam spectrophotometer for the change in absorbance could be masked by endogenous hemoproteins, normal constituents of the respiratory chain. A drawback of the method is its specificity towards NO; to this end, N^G-monomethyl L-arginine (L-NMMA), a specific inhibitor of NOS, should be used in order to determine "true" NO production by mitochondria. Only the portion of oxyMb oxidation sensitive to L-NMMA represents NO production (20).

4.3. L-citrulline assay

In L-citrulline assay, NOS activity is detected based on the conversion of radiolabeled ^{14}C-L-arginine or ^{3}H-L-arginine to L-citrulline (49-50). This assay has been challenged because of the crosstalk between NO and the urea cycle in organs where a significant activity of this cycle is present, such as liver and kidney (36). To eliminate this effect, it is required to introduce in the isolation of mitochondria procedure, several washes with high ionic strength solution (e.g., potassium chloride) to remove arginase from the outer mitochondrial membrane. The efficiency of these washes needs to be checked by following urea production from arginase (22, 51). As an alternative (or in addition), inhibitors of arginase could be added to the medium when urea cycle enzymes (or aborted cycles) are presumed to be present.

4.4. Fluorometric assays

Fluorometric probes are powerful tools to detect NO production by NOS. 5-diaminofluorescein (DAF-2) is converted to a fluorescent compound (DAF-triazole) in the presence of NO. The fluorescence of the triazole has been used to temporally and spatially visualize NO production (52-56). However, the triazole is not obtained from the direct reaction of NO (only 18% seems to be attributed to this reaction; 52), but rather from a reaction (with no clear stoichiometry) between DAF and possibly N_2O_3 (52). It is also not clear if the triazole is actually formed at the site of NO production—indicating co-localization of NO production and the product of the sensor molecule, or if the triazole, formed somewhere within the cell, diffuses to mitochondria ought to the hydrophobicity of this compound, or if the triazole is formed at the mitochondrial membrane due to the favored partition of NO in hydrophobic media. The strength of this assay might be the possibility of imaging NO production in real time. A recent study reported that the specificity problem could be ascribed to the reaction of DAF with dehydroascorbic and ascorbic acids (57). The assay is still valuable if it is used with the combination with the other NOS assays and with the previsions indicated above for real-time bioimaging (58-60).

4.5. Electrode microsensors

Electrode microsensors allow the direct detection of NO, and they can be visually set in the proximity of mitochondria under phase-contrast microscopy. Two types of microsensors have been used for NO detection: amperometric (61-65) and porphyrinic sensors (66). The detection of NO release in brain tissue was first reported by Shibuki in 1990 using an amperometric sensor (63). Kanai *et al* demonstrated NO production by isolated mitochondria using a porphyrinic microsensor tip (66). The drawback of this methodology is that it can only measure the concentration of NO resultant from its rate of production and consumption. Therefore, it is not feasible to evaluate the rate of NO production accurately.

5. CALCIUM SIGNALING AND ACTIVATION OF mtNOS

Intracellular calcium signaling has a crucial role in mitochondrial metabolism. Its functional importance has been extensively investigated in various situations, e.g. involvement in programmed cell death by promoting the mitochondrial permeability transition pore (MPT; 67-68). Calcium is required to activate eNOS and nNOS through the binding to calmodulin (12). Like other constitutive NOSs, calcium has been found as one of the important cofactors for mtNOS activity (22), and its requirement for the activity is concentration dependent (20).

Calcium uptake in mitochondria is mediated by the calcium uniporter. This uptake is electrogenic, driven by the mitochondrial membrane potential. An antiporter that exchanges calcium for protons or sodium, depending on the tissue, mediates the efflux of calcium. It has been proposed that uptake of calcium by respiring mitochondria

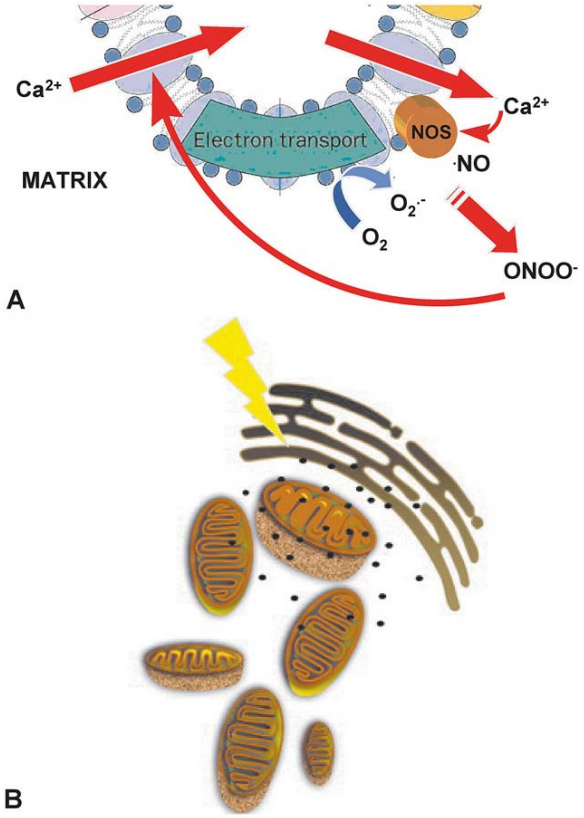

Figure 1. Models for calcium-mediated activation of mtNOS. A: Upon calcium influx, activation of mtNOS leads to the formation of NO. The radical recombination of NO with superoxide anion—the latter species derived from the electron transport chain—results in the formation of peroxynitrite. This species has been proposed to stimulate the efflux of calcium from mitochondria. As calcium levels decrease, a deactivation of mtNOS ensues. B: Calcium, released from ER pools, activates mtNOS in mitochondria located closer to ER. The uptake of calcium by mitochondria and ER creates a gradient of calcium; mitochondria beyond these microdomains, are exposed to less calcium and are able to activate the calcium-dependent dehydrogenases. This differential activation is based on the different calcium EC_{50} for Krebs' cycle and mtNOS, and the mitochondria-ER crosstalk upon stimulation.

may lead to increased peroxynitrite formation in mitochondria (Figure 1A; 20). Peroxynitrite seems to cause calcium release (69) *via* the pyridine nucleotide-dependent pathway (70-71) followed by mtNOS deactivation (Figure 1A). These observations have been interpreted as part of a feedback loop that prevents calcium overloading and allows its release, preserving membrane potential (20). The occurrence of this mechanism requires a significant production of peroxynitrite, likely to happen during State 4 when the mitochondrial production of superoxide anion is significant. However, the release or efflux of calcium from mitochondria has not necessarily been temporally associated with State 4, and considering the high reactivity of peroxynitrite, it is difficult to reconcile a specific chemistry between this species and the calcium antiporter as suggested before (69). The conditions for the applicability of this model seem to be more compatible with stress situations (72).

The affinity of the uniporter has been reported as higher than 5 microM (73), thus, high cytosolic calcium concentrations are required to cause a significant mitochondrial calcium uptake. Consequently, close physical proximity between calcium-releasing domains (such as calcium channels, ryanodine receptors, or IP3 receptors) and mitochondria is required for significant mitochondrial calcium uptake (74-76). It has been demonstrated that the response of mitochondria to calcium uptake is highly heterogeneous upon cell activation (77). Mitochondria in proximity to the calcium-releasing domains uptake a significant amount of calcium (Figure 1B; 76, 78), whereas those located far from these sources, take up relatively less (Figure 1B; 76). Interestingly, elevation in cytosolic calcium concentrations higher than 1 microM has been shown to cause an enhanced association of mitochondria with ER (79), and to arrest mitochondrial motility (80). This arrest of mitochondria movement by calcium may provide assistance with calcium clearance by supporting both ATP-dependent calcium pumps and calcium buffering (80). Mitochondria at sites of high cytosolic calcium could uptake enough calcium to stimulate the calcium-dependent dehydrogenases and mtNOS, resulting in a 30 to 40% inhibition of the oxygen consumption. These mitochondria will continue to produce ATP, although at a slower rate, limiting calcium diffusion to other sites of the cell by supporting the energy needs of calcium-ATPases. The slower oxygen consumption at these foci may delay the onset of anoxia, allowing oxygen to diffuse to these sites, preventing the known overproduction of reactive oxygen species that results from the reoxygenation of a fully reduced electron transport chain. Mitochondria, far from these foci, will be exposed to enough calcium to support the activation of the dehydrogenases, and the consequent ATP production. The availability of oxygen to these latter sites will be favored by the nitric oxide-mediated smoothing of the oxygen gradient.

Various pathophysiological conditions need to be assessed to evaluate the relevance of these two mechanisms —depicted in Figure 1— in terms of the regulation of cellular oxygen consumption by calcium. In this regard, two opposite phenomena via calcium signaling affect the oxygen consumption by mitochondria. On the one hand, calcium activates mtNOS activity and mtNOS-produced NO inhibits cytochrome *c* oxidase in a competitive manner, resulting in the decrease in oxygen consumption (22, 81-82). On the other hand, calcium activates the citric acid cycle by enhancing the activity of three key metabolic enzymes, pyruvate dehydrogenase, NAD^+-dependent isocitrate dehydrogenase, and 2-oxoglutarate dehydrogenase or alpha-ketoglutarate dehydrogenase (67-68), increasing NADH production and by mass action, the oxygen consumption. Therefore, it is important to elucidate how these two pathways are connected when calcium levels arises in the cytosol.

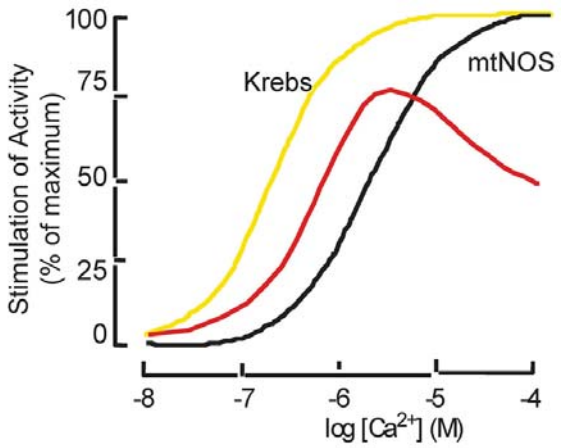

Figure 2. Activation of Krebs' cycle and mtNOS by calcium. The activity increases for Krebs' cycle and mtNOS were calculated considering the EC_{50} for calcium and Vmax for both alpha-ketoglutarate dehydrogenase (as one of the calcium-dependent dehydrogenases of the Krebs' cycle; yellow line) and mtNOS (black line). The resultant of both activations (red line) were calculated based on the stimulation of oxygen consumption by calcium in the presence of NMMA and the inhibition of oxygen consumption by NO by using published data (51, 83).

Using differential experimental settings is one of the excellent methods to evaluate the contribution of these two pathways. If oxygen consumption is followed, supplementation of mitochondria with L-Arg will result in the effect of calcium on mtNOS and Krebs' cycle, whereas the supplementation with NMMA, the specific inhibitor for NOS, only inhibits the function of mtNOS, not Krebs' cycle, eliminating the effect of mtNOS. The subtraction of oxygen consumption of the latter from the former can represent the substantial effect of calcium on mtNOS activity. This strategy successfully showed that the calcium requirement under normoxic condition (220-250 microM oxygen) is different for the calcium-dependent dehydrogenases (0.16 microM) and for mtNOS (0.9 microM; 51). Similar conclusions were observed under hypoxic conditions (or normoxic conditions for mitochondria; 30 microM oxygen; 83), although mitochondria stored 3-times more calcium than under 220 microM oxygen.

The activity of Krebs' cycle (represented by the activation of alpha-ketoglutarate dehydrogenase) and mtNOS are increased as calcium concentration is increased (Figure 2). The stimulation resultant of cell respiration is increased with calcium, but as mtNOS activity is activated at higher concentrations of calcium, the overall oxygen consumption decreases. Thus, the effect of calcium on cell respiration has to be revisited considering the effect on matrix dehydrogenases, NO production, and the actual [NO]/[oxygen] ratio due to the competitive nature of the cytochrome *c* oxidase regulation.

6. MOLECULAR TARGETS OF mtNOS

It is of significance to explore how mtNOS affects downstream targets. Two targets have been reported to date: guanylate cyclase, and cytochrome *c* oxidase. First, the receptor for NO is a soluble isoform of guanylate cyclase (sGC; 81-82). NO binds to the prosthetic heme moiety of sGC, a heterodimeric hemoprotein. Then the NO-activated sGC catalyzes the conversion of GTP to the second messenger molecule cGMP and causes a variety of physiological events (84-86).

Second, the direct binding of NO to cytochrome *c* oxidase has been proved (81-82). The inhibitory effect of NO on mitochondrial oxygen consumption is caused by the competition between NO and oxygen for a binding site at the binuclear center of cytochrome *c* oxidase (40). A recent report provided evidence for a direct interaction of the PDZ domain of mtNOS with a subunit of cytochrome *c* oxidase (31). Our lab has found the same interaction and extended this protein-protein interaction to other subunits of cytochrome *c* oxidase in the presence of calcium (Haynes and Giulivi, submitted manuscript). The evidences provided by our others' research indicate that cytochrome *c* oxidase is the most important molecular target of NO produced by mtNOS (87).

7. SUBCELLULAR LOCALIZATION OF mtNOS

In addition to the biochemical features of mtNOS, some of its biological aspects have been well examined: its subcellular distribution and epigenetic modification. Concerning subcellular location, several studies showed that mtNOS is a membrane-bound enzyme. The initial histochemical study proposed that mtNOS was bound to the inner mitochondrial membrane based on the co-localization of NOS antigen and succinate dehydrogenase (16). Electron microscope analysis using gold particles immunolabelling supported this fact (17). The comparison of the rates of submitochondrial particle (SMP) and whole mitochondrial indicated that most of the NOS activity was detected in the former fraction (21-22). These results leaded to the conclusion that mtNOS was localized at the inner mitochondrial membrane in rat liver.

Several lines of studies have elucidated epigenetic modification of mtNOS, which might affect the subcellular localization of mtNOS. To date, the two posttranslational modifications of mtNOS have been reported are the following: acylation with myristic acid and phosphorylation at the *C*-terminus (88). The former modification could determine the distribution of mtNOS.

Our laboratory presented different acylation pattern for mtNOS from that of eNOS. In our research, the blocking of the *N*-terminal group in mtNOS during the experimental isolation procedure and subsequent SDS-PAGE analysis was demonstrated by the unfeasibility of the Edman protein sequence analysis which requires a free *N*-terminal amino group. The published discrepancies in molecular sizes of mtNOS (besides the general lack of

proteolytic inhibitors use during purification and isolation procedures when using biological materials), the identity of mtNOS as nNOS alpha, and the reported acylation pattern may result in an altered electrophoretic mobility pattern. To verify the acylation of mtNOS, samples of purified mtNOS were precipitated with acetone, and washed with organic solvent to remove any unspecifically bound lipids. After the treatment of this sample with alkaline methanolysis, mass spectrometry was performed to identify the fatty acids. The analysis showed mtNOS was acylated with myristic acid via an ester bond (88). No evidence for palmitoylation of mtNOS was found in this study.

The co-/post-translation modifications have been observed mainly in eNOS. The double acylation, myristoylation and palmitoylation, modifies eNOS, and the biological importance have been extensively discussed (89-95). Palmitoylation of iNOS has been recently reported only in mouse myoblast cell line (96). No acylation has been found for nNOS to date. Therefore mtNOS is unique in terms of the fact that it is only myristoylated via an ester bond (88). It is noteworthy that mtNOS was myristoylated via an ester bond, not via an amide linkage (88). Two types of protein myristoylation have been found until now: *N*-myristoylation in which the fatty acid is linked via a hydroxylamine-resistant amide linkage (97) and *E*-myristoylation via a hydroxylamine-labile thioester linkage (98-99).

The peptide sequence of mtNOS does not have a consensus sequence for myristoylation site like (MGXXXS/T; 100-101), indicating that mtNOS was not myristoylated at an *N*-terminal glycine residue (88). Myristic acid is bound to mtNOS via an ester bond in our study, not through an *N*-terminal Gly (88); however, the specific residue for the ester linkage has not yet been determined. Much of the modification mechanism and biological significance of mtNOS myristoylation remains unknown. *N*-myristoylation seems to be mainly co-translational (12, 102-104), whereas *E*-myristoylation can occur post-translationally (98).

Concerning the functional importance, *E*-myristoylation could increase hydrophobicity of mtNOS and contribute to a stronger anchoring of mtNOS to inner mitochondrial membrane. In principle, fatty acid acylation, like myristoylation and palmitoylation, functions as important lipid-membrane anchors (101). For example, both fatty acyl modifications of eNOS are essential for its binding to caveolae membrane (92). Without these modifications, eNOS cannot properly localize to the membrane and act as a membrane-bound enzyme (105). iNOS and nNOS are predominantly cytosolic proteins probably because of the lack of acylation (102, 106). Extrapolating the acylation roles from other NOSs, it could be suggested that *E*-myristoylation of mtNOS is required for a proper localization of mtNOS at the mitochondrial membranes given that hydrophobic (and positively charged) compounds tend to partition to mitochondria.

The *N*-terminal PDZ domain of mtNOS can be synergistic with the targeting given by *E*-myristoylation. In contrast to the previous notion that nNOS is mainly soluble owing to the lack of acylation, some authors showed that a substantial portion of nNOS was found in the particulate fraction (107). Moreover, it has been reported that the interaction between PDZ domain of nNOS and its counterpart proteins, PSD (postsynaptic density)-95, PSD-93 or skeletal muscle alpha1-Syntrophin, mediate the membrane association of nNOS (108). This interaction regulates the distribution and activity of the enzyme (109). Considering that mtNOS is identified as nNOS alpha bearing a PDZ domain (88), it is assumed that specific protein-protein interaction via PDZ domain might play a role in membrane targeting and/or protein-protein interaction of mtNOS. In this regard, a recent study indicated a direct interaction between the PDZ domain of mtNOS and one of the subunits of cytochrome *c* oxidase (31). Future research is required to explore the targeting mechanisms of mtNOS because it would affect the regulation of cytochrome *c* oxidase activity, respiratory rate and oxygen consumption (110).

8. mtNOS PHOSPHORYLATION

Phosphorylation at the *C*-terminus has also been discovered in mtNOS (88). The presence of phosphatase inhibitor during the mtNOS sample preparation allowed detecting a phosphorylated fragment by MALDI-TOF analysis. It was suggested that the phosphorylation occurs at a Ser residue for the fragment lacks Tyr or Thr residues. From the three possible Ser residues present in this fragment, Ser-1412 in mtNOS is considered as the most likely site of phosphorylation, based on the alignment with human and bovine eNOS peptide sequences and the deduction from Akt phosphorylation motif. The phosphorylation of Ser-1177 in human eNOS and that of Ser-1179 in bovine eNOS is the important regulatory factor to control the enzyme activity (111-114). The significance of this phosphorylation of mtNOS has been unknown, but phosphorylation at the *C*-terminus might be important to modulate the mtNOS activity and/or protein-protein interactions.

9. mtNOS vs. NITRIC-OXIDE ACTIVITY IN MITOCHONDRIA

Early studies showed immunoreactivity of mitochondria to anti-eNOS (16-18) in rat liver, brain, heart, kidney and skeletal muscle and to anti-nNOS (19) in human skeletal muscle. Solid evidence that mtNOS is one of the nNOS isoforms was provided by two independent groups using advanced protein technology and genetically modified murine models.

First, our laboratory provided the evidence that the mtNOS had an identical protein sequence to nNOS alpha using MALDI-TOF method for peptide mass fingerprinting (PMF; 88). To this end, purified mitochondria were subjected to two-dimensional electrophoresis to obtain a higher resolution in the separation of co-migrating proteins, followed by in-gel digestion and peptide sequence of the fragments. Trypsin- or endoproteinase V8-digested fragments had a 100%

homology to rat nNOS isoform. Because four splice variants of full-length of nNOS (nNOS alpha) had been reported (nNOS beta, nNOS mu, NOS gamma and NOS-2), further work was done to identify which variant was closest to mtNOS. nNOS gamma and nNOS-2 variants were excluded since mtNOS had no fragments consistent to these isoforms. RT-PCR of mRNA from liver rat mitochondria with validated controls eliminated the possibility of nNOS beta and nNOS mu based on the amplified size of the PCR products. Moreover, it was proved that the amplified product specifically matched most of the transcript sequence that only nNOS alpha contained.

Second, experiments using genetically engineered murine model lacking the three isoforms of NOS strengthened the above conclusions (66). Kanai *et al* reported that no mtNOS was detected from nNOS-deficient mouse whereas mtNOS was detected in both eNOS- and iNOS-deficient mice. Only nNOS alpha variant was missing in the murine model because the targeting construct removed only exon-2 containing PDZ domain (115), which is the unique domain for the nNOS alpha. The other nNOS variants were expressed in the murine model. This result indicated that mtNOS in mouse heart was the product encoded by the nNOS alpha gene. These results have clearly ruled out the possibility that a unique gene encodes specifically for mtNOS, or that mtNOS is a new isoform or variant of NOS, regardless of the species (mouse, rat) or organ (heart, liver).

Despite these reports, many conflicting results about mtNOS isoform have been published. As an example, a recent report indicated that mtNOS was none of the three NOS isoforms based solely on the immunoreactivity of antibodies to NOS (116). According to Boveris et al, two forms of mtNOS exist; mtNOS-1 (145 kDa) localized in brain whereas mtNOS-2 (130 kDa) is in liver, thymus and kidney (117). This classification is based only on crossreactivity to isoform-specific antibodies without considering tissue-specific posttranslational modifications of NOS.

Experimental settings and understanding of the technique limitations are the basis for most of these observations regarding identification of mtNOS. The three main reasons are the following: (*a*) cross-reactivity of isoform-specific anti-NOS antibodies, (*b*) different sources of mitochondria, and (*c*) purification of mitochondria. Many reports have used isoform-specific anti-NOS antibodies in order to differentiate the NOS isoforms. However, it is well known that the specificity of antibodies (even monoclonal ones) is not always reliable for depends on the antigenicity of the epitope and the binding constant of the antigen with the antibody. In this regard, multiple bands in western blots are detected by anti-nNOS, anti-eNOS, and anti-iNOS antibodies when they supposedly should react only with a certain isoform of NOS (36). Epitopes all three NOS isoforms of the antibody have high homology to NADPH-cytochrome P450 reductase (118). In our hands, anti-nNOS antibody from BD Biosciences Pharmingen (clone 16) crossreacted strongly with other NADPH-binding proteins such as glutamate dehydrogenase: (GDH; P10860), C1-Methylenetetrahydrofolate dehydrogenase (C1-THF synthase; P27653), and 10-formyltetrahydrofolate dehydrogenase (10-FTHFDH; P28037). These proteins were identified by amino acid sequencing performed with Edman degradation of trypsin fragments and/or MALDI-TOF of the tryptic fragments. The two major bands, 130 kDa and 60 kDa, with the immunoblot with anti-nNOS antibody, were identified as nNOS and GDH by MALDI-TOF analysis; *N*-terminal amino acid sequencing revealed the minor bands as C1-THF synthase and 10-FTHFDH. This antibody was produced using the NADP-binding site (immunogen 1095-1289) of human nNOS (P29475). In the case of GDH, the NADP-binding site of nNOS has 46% identity in eleven amino acids to that of rat GDH, which would be the cause for the observed crossreactivity.

These facts validate the limited support that the sole use of antibodies provides to the identification of any NOS. Although more time consuming and labor intensive, it is accurate to analyze the actual peptide sequence as it is appropriate for any protein chemistry work. Given that mtNOS is not an abundant protein, advantage of cutting edge technologies—like peptide mass fingerprinting analysis— should be taken.

The second reason is tissue specificity of mtNOS. Although mtNOS was identified as nNOS alpha by the most reliable analysis like peptide mass fingerprinting, the experimental source of mtNOS was still limited to certain organs in specific species (i.e., mitochondria from rat liver, pig and dog heart), although the transcript analysis by RT-PCR indicated the existence of mtNOS in tissue of many organs (liver, brain, heart, muscle, kidney, lung, testis and spleen; 88).

Recently, it has been proposed that a mammalian ortholog of AtNOS1 is one of candidates for mtNOS (119). AtNOS1 is identified as a plant NOS gene which is involved in the hormonal signaling at first (120; Gene ID #AT3G47450.1). The overexpression of the mouse ortholog of this gene in COS-1, an African green monkey kidney fibroblast-like cell line, showed that the mouse ortholog of AtNOS-1 was transported to inner mitochondrial membrane. Although the crucial evidence to identify mouse ortholog of AtNOS-1 as one of mtNOS is still waiting to be uncovered, it should be always taken into account the possibility that mtNOS may exist in various forms depending on different organs and developmental stages.

The last reason is the contamination with NOSs other than mtNOS. For example, NOS from Kupffer cells (hepatic macrophages originated from bone marrow) are endowed with iNOS (121-122) and given that there is always a background of activated cells, its contribution—if not avoided—cannot be dismissed (12). Thus, an increase in mitochondrial NOS activity cannot necessarily be attributed to a change in mtNOS expression, but entirely caused by an overexpression—even a quantitatively minor one—of iNOS, especially in certain clinical situations such as septic shock. Thus, isoforms of NOS may be falsely recognized as mtNOS when mitochondria are crudely

prepared and the actual physiological situations not tested in detail.

Recently, the association between eNOS and the outer mitochondrial membrane has been reported in human embryonic kidney (HEK) cells (123). In this study, adenoviral vector system was used for the overexpression of eNOS (123). The binding was found by an *in vitro* study using cell lines human umbilical vein endothelial cells, and a non-endothelial cell line, human embryonic kidney cells (123). Several experimental issues might be considered when conclusions are raised in terms of identity of mtNOS and its localization. First, the *in vitro* conditions may be different from physiological ones; overexpression of proteins might lead to localizations different from those found *in vivo*. Second, although the authors described that a pentabasic amino acid sequence was essential for targeting to mitochondrial outer membrane, this length is not enough for protein targeting to these organelles. In general 17-35 amino acids in length are required as a leader sequence for proper translocation to mitochondrial membranes (124-125). Third, post-translational modifications, such as acylation, which may be relevant for protein targeting and/or activity, may not have been present during the overexpression of eNOS, resulting in altered localization and/or activity. Lastly, the association of eNOS with cytochrome *c* oxidase would not be feasible because lacks the PDZ domain required for this binding.

If the binding of eNOS observed *in vitro* holds true *in vivo* situations, eNOS bound to outer mitochondrial membrane may have a distinct function in the cells where no mtNOS was found (31). For example an immunohistochemical study showed that eNOS in rat and mouse sensory neurons was targeted to juxtamitochondrial smooth endoplasmic reticulum (126), suggesting NO generated by this eNOS could diffuse to the inner mitochondrial membrane. The possible explanation would be that these two are complementary phenomena for a tight regulation of respiration (127).

Studies from our lab were performed with purified rat liver mitochondria with very low level of non-mitochondrial contamination after differential centrifugation and Percoll centrifugation. Therefore, only when experiments are done with isolated and purified hepatocytes, the contribution from iNOS can be ignored, and solely mtNOS becomes feasible to detect. Consequently further investigations will be required to verify the possible variation of mtNOS from various sources (different tissue, different organism) not by isoform-specific antibodies, but by more reliable method like peptide mass fingerprinting or gene-targeted mouse models.

10. mtNOS IN NO-RELATED BIOLOGICAL PHENOMENA

It might be possible that different sources of NO have different roles in a given organ during aging or development, or in different tissues at a given period. Cytosolic NOS activity is increased as mtNOS activity in brain is decreased during rat aging, possibly exhibiting a compensatory activity (25). Mature neurons express cytosolic NOS, whereas mtNOS is predominant in immature neurons (59). mtNOS from liver primary cell culture decreases to negligible values after 4 to 5 days of culture (Unpublished data, Haynes, V. and Giulivi C, 1999). *In vivo* mtNOS expression and activity increased at late embryonic and early postnatal stages, followed by a decrease in the adult stage (25). These evidences imply that mtNOS activity could be sequentially altered in accordance with mammalian developmental stages.

It will be important to investigate the extent of NO—generated from mtNOS—that contributes to biological reactions beyond mitochondria, and to define the coordination of mtNOS and NOSs expression. mtNOS has been detected from many different organs, similar to nNOS, eNOS, and iNOS. Its ubiquitous distribution implies that mtNOS might play important roles in regulating many biological phenomena through the body. Several physiological conditions that affect mtNOS activity have been reported to date. For instance, mtNOS activity might be upregulated by certain situations like hypoxia (128), cold acclimation (129), or high altitude (32). Moreover, the increase in mtNOS activity might be related to the effect of some pharmaceutical agents. mtNOS activity and protein expression are increased in rat kidney, heart, and liver with the treatment of enalapril angiotensin-converting enzyme (28-29, 130-131).

In contrast, several situations may be related to the decrease in mtNOS activity in rat heart, kidney, and brain (24, 30, 130-133). Decreases in mtNOS activity were described in liver, kidney, brain, and heart of 2-yr old rats (131-133). However, in a different study, similar percentages of mtNOS activity were reported but found statistically insignificant (130). Although such a decline might seem physiologically important, given that also the oxidative phosphorylation capacity of mitochondria declines with age, this decrease might reflect other changes described for mitochondria during aging. Furthermore, interpretation of these results needs some cautions. It is important to verify whether the measured NOS activity is truly originated from the mtNOS, and to eliminate the effects by other NOSs. In certain situations, especially in drastic metabolic changes like cold acclimation, NOS activity from other sources might be responsible for these changes.

11. mtNOS changes under pathophysiological conditions

As described above, the mtNOS plays important role in cellular respiration in physiological situations. Studies have provided evidence that certain pathological situations affect mtNOS activity in some tissues (26, 134-137). mtNOS activity was increased (122%) in diaphragm and (32%) in heart in lipopolysaccharide-treated rats (134-135); an increase (270%) of mtNOS was described in skeletal muscle during hypothyroidism (136). In contrast, marked decreased levels of mtNOS expression and activity were described in adenocarcinoma (137). A study using a

hyperammonemia rat model also demonstrated decreased mtNOS activity with a hippocampal mitochondrial dysfunction (26). More work is required in the future to clarify the contribution of NOSs other than mtNOS to the reported changes in activity.

mtNOS may be profoundly involved in the cell growth or cell death. A study demonstrated that mtNOS regulates the switch between proliferative and quiescent status of rat liver *via* redox signaling (138). In a series of studies performed with thymocytes, it was suggested a role for mtNOS in apoptosis (33-35). NO has two paradoxical effects on programmed cell death: pro- and anti- apoptotic reactions in cells (139-142). Concerning the pro-apoptotic effect of NO, mitochondria have been identified as a key organelle for apoptosis, thus it could be easily speculated that mtNOS might control this event. The concept that NO triggers apoptosis has been established by many studies (143-144). NO induces apoptosis via activation of the mitochondrial permeability transition pore (MPT; 143). It has been suggested that the release of cytochrome *c* from the mitochondria to the cytosol is key for this process (144). Oxygen- and nitrogen-reactive species have been proposed to have a role in apoptosis. For instance, the increase in hydrogen peroxide (H_2O_2) precedes cytochrome *c* translocation from mitochondria (145). Therefore, it will be important to determine the link among mtNOS, cytochrome *c* and MPT induction or in what mechanisms mtNOS might be involved in the pro-apoptotic reaction.

On the contrary, NO has a role as anti-apoptotic molecule under certain situations (146-148). An anti-apoptotic effect by NO has been described by its preventive effect on hepatocytes after reperfusion (146). NO treatment of hepatocytes after storage could improve the viability of transplanted livers. Therefore, it might be meaningful to control NO function for clinical applications. Although it has been unknown the extent that mtNOS contribute to this phenomenon, further studies on the mtNOS involvement on apoptosis would be of significance.

12. TRANSCRIPTION/TRANSLATION REGULATION OF mtNOS

One of the unsolved questions about mtNOS is how the complex nNOS transcription / translation process is regulated. nNOS has diverse structures and promoter uses, which makes the isoform identification of mtNOS more intricate. The transcription and translation process of nNOS gene is one of the most complicated pathways in biology (149-150). First, four alternative splice variants have been known, lacking PDZ domain, which are multifunctional motifs that mediate protein-protein interactions by binding to the *C*-terminus of the counterpart protein (151). The distribution of these nNOS variants might be tissue-specific and tightly regulated (152), especially in brain. Alternative patterns of RNA splicing are commonly observed in vertebrate nervous systems because multiple isoforms of proteins are essential to create the complexity of neuronal development and function (153).

In addition to the alternative splice events, the use of alternative promoters has been observed in nNOS gene transcription (154). Nine alternative first exons of nNOS, driven by separate promoters, have been found in the human gastrointestinal tract (149-150). In most cases, the different use of alternative promoters could affect the transcriptional patterns and translational efficiencies (155). It is worth noting that the variant alternative promoters do not usually result in alternative splice variants because they use the same translation initiation site and the same open reading frame. In the processing of human nNOS protein expression, nine distinct first exons are spliced to the common exon 2 of nNOS that has the initiation codon. Similarly, the process for nNOS gene expression has the same mechanism in rodents (108). These multiple forms of exon 1 are selectively used in a tissue-specific manner (154). Given that mtNOS is a nNOS alpha, complexity in its transcription / translation process could be intricate and might be different depending on tissue type. This may contribute to the difficulties of some labs with the isoform identification. Finally, the unknown questions about the regulatory mechanisms of mtNOS transcription are waiting to be resolved: (*a*) how alternative promoters are utilized for mtNOS expression?; (*b*) is the selective use tissue-specific?; (*c*) does the difference of alternative promoters make any difference of the alternative splice variant? Answering these questions will validate significance of mtNOS in physiopathological situations.

13. CONCLUSIONS

mtNOS has a biological importance on cellular respiration, and is a key enzyme for many pathophysiological reactions that are affected by mitochondrial respiration. The broad distribution of mtNOS may imply its impact on aerobic organs. To date, many important features of mtNOS have been recently revealed by our and other labs as discussed above. Nevertheless, much of the physiopathological significance of mtNOS waits to be clarified. For instance, it is still unknown how mtNOS activity coordinates with the expression of other NOS. The expression changes of mtNOS in different organs and during various developmental stages have not been well understood yet. Furthermore, most of the controlling mechanisms of mtNOS expression *in vivo*, such as transcriptional regulation or epigenetic modifications, are unclear. Thus, more detailed studies will be necessary for more comprehensive understanding of mtNOS roles.

14. ACKNOWLEDGEMENTS

This study has been supported by NIH (ES012691).

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Abbreviations: NO: nitric oxide; mtNOS: mitochondrial nitric-oxide synthase; nNOS: neuronal nitric-oxide synthase; iNOS: inducible nitric-oxide synthase; eNOS: endothelial nitric-oxide synthase; EPR: electron paramagnetic resonance; DAF-2: diaminofluorescein-2; PMF: peptide mass fingerprinting; oxyMb: oxymyoglobin; metmyoglobin: metMb; L-NMMA: N^G-Monomethyl-*L*-arginine; NADPH: nicotinamide adenine dinucleotide phosphate; FAD: flavin adenine dinucleotide; FMN: flavin mononucleotide; MPT; mitochondrial permeability transition; sGC: soluble isoform of guanylate cyclase; SMP: submitochondrial particle; MALDI-TOF: matrix assisted laser desorption /ionization- time of flight; PMF: peptide mass fingerprinting

Key Words: Mitochondria, Mitochondrion, Mitochondrial Nitric Oxide, mtNOS, Calcium Signaling, Peroxynitrite, Cellular Respiration, Apoptosis, Review

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