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Mitochondrial Nitric-Oxide Synthase: Enzyme Expression, Characterization, and Regulation

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Nitric oxide is generated in vivo by nitric-oxide synthase (NOS) during the conversion of L-Arg to citrulline. Using a variety of biological systems and approaches emerging evidence has been accumulated for the occurrence of a mitochondrial NOS (mtNOS), identified as the alpha isoform of neuronal or NOS-1. Under physiological conditions, the production of nitric oxide by mitochondria has an important implication for the maintenance of the cellular metabolism, i.e. modulates the oxygen consumption of the organelles through the competitive (with oxygen) and reversible inhibition of cytochrome c oxidase. The transient inhibition suits the continuously changing energy and oxygen requirements of the tissue; it is a short-term regulation with profound pathophysiological consequences. This review describes the identification of mtNOS and the role of posttranslational modifications on mtNOS' activity and regulation.

KEY WORDS: Nitric oxide; nitric-oxide synthase; mitochondria; posttranslational modifications; oxygen consumption; oxygen.

INTRODUCTION

Nitric oxide is formed from L-arginine by nitric-oxide synthase (NOS), which oxidizes the guanidino nitrogen of arginine, releasing nitric oxide and citrulline (Bredt and Snyder, 1990, 1994). Three main NOSs are expressed in mammals and differ in their functions, amino acid sequence, posttranslational modification, and cellular location. Two NOS, neuronal NOS (nNOS or NOS-1) and endothelial NOS (eNOS or NOS-3), are constitutively expressed and involved in signal cascades (Bredt and Snyder, 1990; Mayer *et al*., 1989, 1990). The third NOS is cytokine-inducible (iNOS or NOS-2) and functions as both a regulator and effector of the immune response (Förstermann *et al*., 1992; Stuehr and Marletta, 1987). The consequence to this diversity of location and function is a specific regulation of each isoform. For example, NOSs differ significantly regarding Ca^{2+} levels required to bind calmodulin, which triggers heme reduction and nitric-oxide synthesis (Abu-Soud *et al*., 1994; Panda *et al*., 2001). They also have different capacities to be up- or downregulated by Ser/Thr phosphorylation (Fulton *et al*., 1999; Harris *et al*., 2001). Using rat liver, we provided unequivocal evidence for a localization of a NOS (mitochondrial NOS or mtNOS) at the inner membrane of mitochondria (Giulivi *et al*., 1998; Tatoyan and Giulivi, 1998). Given NOS' diverse biochemical characteristics, it could be postulated that the production of nitric oxide by mitochondria is highly regulated because of the critical role that this molecule has on cellular respiration (Brown and Cooper, 1994; Cleeter *et al*., 1994; Poderoso *et al*., 1996). In the following sections, the identification of mtNOS and the role of posttranslational modifications on its activity and regulation are summarized and discussed.

PRODUCTION OF NITRIC OXIDE BY MITOCHONDRIA

Our studies were the first providing evidence for production of nitric oxide by *purified* mitochondria (Giulivi, 1998; Giulivi *et al*., 1998, 1999; Tatoyan and Giulivi, 1998; French *et al*., 2001). This production of nitric oxide was demonstrated by using direct (L-citrulline production, evaluated by using colorimetric or radiolabeled

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compounds) and indirect techniques (nitric-oxide generation, evaluated by electron paramagnetic resonance with spin trapping; nitric oxide-dependent oxidation of oxymyoglobin). More evidence was furnished by the measurement of NOS activity in mitochondria isolated from purified hepatocytes, (abrogating the putative contamination of Kupffer cells), mitoplasts (mitochondria stripped of the outer membrane). Isolation and purification of mtNOS, mainly localized at mitochondrial membranes, allowed obtaining critical kinetic constants and dependence on cofactors and cosubstrates (Giulivi, 2003; Tatoyan and Giulivi, 1998).

Other labs had reached similar conclusions or extended the knowledge in this field in terms of the occurrence of a mtNOS by using less purified mitochondrial fractions, isolated cells, and various approaches such as colocalization of mitochondria with a production of nitric oxide in intact cells, nitric-oxide detection by electrode in wild-type and nNOS-KO mice mitochondria, among others (Bates *et al*., 1995, 1996; Dedkova *et al*., 2004; Ghafourifar and Richter, 1997; Kanai *et al*., 2001; Kobzik *et al*., 1995; López-Figueroa *et al*., 2000).

Considering that the constitutive forms of nitric-oxide synthase, i.e., nNOS and eNOS, account for the rapid, transient, calcium-dependent production of nitric oxide (Bredt and Snyder, 1990; Mayer *et al*., 1989, 1990), thus, it would be expected that increases in mitochondrial calcium be required to activate mtNOS. In line with this assumption, stimulation of nitric-oxide production by mtNOS was observed by bolus additions of calcium to mitochondria (Ghafourifar and Richter, 1997). The authors proposed that uptake of calcium by respiring mitochondria may lead to increased peroxynitrite formation in mitochondria, which in turn causes calcium release (Schweizer and Richter, 1996) via the pyridine nucleotide-dependent pathway (Lötscher *et al*., 1979) followed by mtNOS deactivation. These observations have been interpreted as part of a feedback loop, which prevents calcium overloading and allows its release preserving membrane potential (Ghafourifar and Richter, 1997).

An apparent discrepancy arises in terms of the role of calcium on the rate of oxygen consumption by mitochondria: on one hand, mitochondrial calcium increases the rate of oxygen consumption as a result of the activation of calcium-activated dehydrogenases (Denton and McCormack, 1985, 1990, 1993; Hansford, 1985; McCormack *et al*., 1990), and on the other, by activating mitochondrial nitric-oxide synthase, decreases the oxygen consumption by inhibiting cytochrome oxidase activity. When we evaluated the rates of State 3 oxygen consumption in the presence of N^{G}-monomethyl-L-Arg (NMMA), a competitive inhibitor of mtNOS, at various concentrations of calcium, the $K_{0.5}$ was 0.1 μM (similar to that required for the activation of the Krebs' cycle) whereas in those with L-Arg (in which mtNOS was saturated with L-Arg) the $K_{0.5}$ was 0.45 μM (Traaseth *et al*., 2004). By plotting the difference between the rates of oxygen consumption in State 3 with L-Arg and with NMMA at various calcium concentrations, a $K_{0.5}$ of 0.3 μM was obtained, similar to the $K_{0.5}$ (0.26 μM) of the dependence of the rate of nitric-oxide production on calcium concentrations, and within the values of other $K_{0.5}$ found for purified nNOS (Bredt and Snyder, 1992; Mayer *et al*., 1989). Thus, the difference between these $K_{0.5}$ indicates that the activation of dehydrogenases, followed by the activation of mtNOS would result in the modulation of the Krebs' cycle activity by the modulation of nitric oxide on the respiratory rates (Traaseth *et al*., 2004). This would ensue in changes in the NADH/NAD^+ and ATP/ADP ratios, which would influence the rate of the cycle and the oxygen diffusion.

BIOCHEMISTRY OF MITOCHONDRIAL NITRIC-OXIDE SYNTHASE

The identification of mtNOS was a critical step in this research because it allowed the assignment of this protein to one of the known isoforms, or to depict it as a novel isoform. By using nitric-oxide electrodes to follow the production of nitric oxide by mitochondria, mtNOS has been identified as the nNOS, for nNOS-KO mice have no mtNOS (Kanai *et al*., 2001). Independently, our lab reached the same conclusions in terms of identifying mtNOS as the alpha isoform of nNOS and expanded this concept to identify the isoform and posttranslational modifications (Elfering *et al*., 2002). Briefly, purified mtNOS was separated by 2D-electrophoresis, followed by in-gel digestion with either trypsin or endoproteinase V8, and MALDI-ToF analyses were performed on the eluted fragments. The resulting sequences were blasted against in-silico trypsin- or V8-digested proteins from the PDB and matched to sequences of constitutive rat nNOS (Elfering *et al*., 2002). Given that mouse bNOS has five isoforms (known as bNOS-1, bNOS2, bNOS-beta, bNOS-gamma, and bNOS-MU or muscle-specific; Brenman *et al*., 1997; Ogura *et al*., 1993; Silvagno *et al*., 1996) produced by alternative splicing of mRNA, the question remained whether mtNOS was one of these products or represented a novel alternative splicing product. Some of the fragments obtained with MALDI excluded NOS-gamma and NOS-2, NOS-beta and NOS-gamma seemed unlikely candidates based on their MW, suggesting that either NOS-1 or -MU was mtNOS. RT-PCR experiments performed on enriched poly(A)$^+$ mRNA from rat liver (using primers based on

MALDI-ToF sequences or gene-specific) and PCR experiments performed on rat liver cDNA resulted in the amplification of segments of the transcript corresponding to nNOS alpha isoform.

Our results combined from MALDI, MW, and p*I* indicated that mtNOS is bNOS, excluding the possibility of a novel isoform or an alternative splicing product. The identification of this enzyme was crucial because it will allow studying its biochemistry in detail with the previous knowledge that we have on the bNOS isoform.

Other studies had emerged perceiving mtNOS as a novel or other than the nNOS isoform as indicated above (Brookes, 2004, and references therein). Distinction needs to be made on what is considered identification parameters for a protein in biochemistry. Protein characterization (not identification) based on Western blotting technique is based on the crossreactivity of an antibody with a small segment of the protein (or epitope), which is usually smaller than the actual antigen used for immunization (usually 1 to 15% of the protein). Thus, a positive result in a Western blot should be understood as the crossreactivity of an antibody with a certain epitope in the protein, not necessarily indicating 100% homology with the rest of the protein. Considering that proteins like eNOS, iNOS, and nNOS have a 49 to 56% homology and that most of the commercially available antibodies are directed to the C-terminal half of the enzyme (or reductase domain) which shows pronounced sequence similarities to cytochrome P450 reductase (Bredt *et al*., 1991) and where most of the homology among NOSs is present (48 to 55%) provides limited evidence for identification purposes. Even the combination of Western blotting with MW calculated from SDS-PAGE (where the error associated with proteins with a MW higher than 100 kDa is expected to be higher than 10% because of the limited availability of high MW standards and the lack of linearity between MW and mobility in this range, aside from the error constituted by the presence of posttranslational modifications such as acylation or glycosylation; Laemmli, 1970; Weber and Osborn, 1969) furnish inadequate information for identification purposes.

POSTTRANSLATIONAL MODIFICATIONS OF mtNOS AND THEIR ROLE IN NITRIC-OXIDE REGULATION

Acylation Pattern of Mitochondrial Nitric-Oxide Synthase

All three NOSs (i.e., n-, e-, and mac-NOSs) were recovered from primary cells in both a soluble and a particulate fraction (Hecker *et al*., 1994; Liu and Sessa, 1994; Pollock *et al*., 1991). Endothelial cNOS, which was recovered from resting cells almost exclusively in the latter fraction (Pollock *et al*., 1991), was labeled when host cells were incubated with radioactive myristic acid (Liu and Sessa, 1994). Here, the amino-terminal group was cotranslationally linked to myristic acid, supported by the following evidences: (1) the inhibition of myristic acid incorporation by a mutation of the amino-terminal Gly indicating that the fatty acid was bound by an amide bond to the protein; (2) cycloheximide treatment abolished the incorporation of myristic acid indicating that the fatty acid was incorporated during protein synthesis; and (3) the identification of myristic acid was performed by the release of myristoyl methyl ester from the protein, after acid methanolysis followed by hydroxylamine treatment. Later, it was reported that eNOS is also palmitoylated at the Cys residues. These acylations allowed the detection of the enzyme close to calveolin, located at the plasma membrane, in intact endothelial cells (Busconi and Michel, 1993). With bNOS isolated from rat but not rabbit, most of the protein sedimented in a subcellular fraction whose marker enzymes were typical of endoplasmic reticulum (Liu and Sessa, 1994). mtNOS was found mainly localized at the inner mitochondrial membrane, requiring the presence of CHAPS to solubilize the enzyme from mitochondrial membranes, indicating that the enzyme is tightly bound to the membrane. Experiments designed to investigate the putative acylation of mtNOS resulted in the finding that myristic acid is linked to mtNOS through an oxy- or thio-ester bond. Myristic acid was probably bound during a reversible, posttranslational process, catalyzed by acyltransferases. It should be noted that the pattern of acylation found with mtNOS differs from that observed with eNOS, in which an *N*-terminal myristoylation and palmitoylation of Cys residues were found. No data on acylation are available for the other two NOS, i.e., bNOS and macNOS; however, their main soluble localization may indicate a low or negligible acylation.

The occurrence of lipid–protein linkages in mtNOS may indicate an alternative modulatory role based on acylation–deacylation processes. The exact function of acylating proteins is still not known, and in this context, protein–protein interactions, membrane localization, or subcellular distribution has been proposed. In this case, it could be speculated that acylation of mtNOS is implicated in the regulation of mitochondrial nitric-oxide production. If acylated mtNOS could be incorporated to the mitochondrial membranes, this localization might be advantageous for the following reasons: first, nitric oxide will be produced closer to the target site, cytochrome oxidase, thus minimizing secondary reactions; second, it will

extend the lifetime of nitric oxide considering that this molecule when is produced in an aqueous, aerated solvent is consumed faster than if it is produced close to or in the membrane because nitrosyl dioxyl—the product of nitric oxide and oxygen—can be stabilized by H bonds in an aqueous milieu (Beckman, 1996); and third, it may facilitate the targeting of the protein to mitochondria given that hydrophobic substrates tend to concentrate in these organelles.

Of note, proteins modified by ester bonds are potentially subject to dynamic regulation: the linkage and cleavage of palmitic acid to proteins are catalyzed by yet uncharacterized palmitoylthiotransferases and palmitoylthioesterase (Resh, 1994). These processes may underlie the ability of the enzyme, as with eNOS, to form stable, but dynamically regulated, associations with cell membranes (Busconi and Michel, 1993). This type of regulation could be also present in mitochondria and added a type of regulation to enzymatic activity by controlling the compartment in which the enzyme is present.

Phosphorylation of mtNOS

In response to agonists, eNOS accumulated phosphate and became soluble (Michel and Busconi, 1993) probably by decreasing the positive charge of a region that contributed electrostatically to the binding of eNOS to lipid. Other studies provided evidence that all three NOS isoforms immunoprecipitated from host cells are phosphorylated (Dawson *et al*., 1993; Michel and Busconi, 1993). It has been shown that kinase- and phosphatase-dependent events occurring in cells modified NOSs activity (Bredt *et al*., 1992; Brune and Lapetina, 1991; Nakane *et al*., 1991). Recently, it has been reported that regulation of eNOS activity involves phosphorylation (Chen *et al*., 1999; Dimmeler *et al*., 1999) and coordinated signaling through Ser-1177 and Thr-495 by multiple protein kinases and phosphatases (Michell *et al*., 2001).

Previous studies performed by our lab indicated that a phosphorylation was present in mtNOS at the fragment comprised between amino acids 1408 and 1421. Interestingly, Ser-1177 or 1179 from human or bovine eNOSs, respectively, which was found to play a critical role in the coordinated phosphorylation/dephosphorylation of the protein (Michell *et al*., 2001), is homologous to Ser-1413 in rat bNOS, suggesting that this position could be subjected to phosphorylation. It is tempting to hypothesize that if ATP and respiratory substrate levels are high, then phosphorylation of mtNOS may enhance NOS' activity (by analogy with eNOS). This regulation will increase the production of nitric oxide, thus allowing an inhibition of cytochrome oxidase and the consequent production of ATP. This pathway will allow that oxygen and other substrates get to cells that not necessarily are close to blood vessels, assuring a homogenous distribution.

PHYSIOLOGICAL ROLE OF mtNOS

We demonstrated that, under physiological conditions, the production of nitric oxide by mitochondria has an important implication for the maintenance of the cellular metabolism, i. e., nitric oxide (produced by rat liver mitochondria) modulated the oxygen consumption of the organelles (Giulivi, 1998, 2003; Giulivi *et al*., 1999). This effect was achieved through the reversible inhibition of cytochrome oxidase by nitric oxide (Giulivi, 2003; Haynes *et al*., 2003, and references therein). This transient inhibition suits the continuously changing energy and oxygen requirements of the tissue. However, if a sustained inhibition of cytochrome *c* oxidase is allowed, then other deleterious effects may happen: inhibition of ATP synthesis, release of cytochrome *c* (Ghafourifar *et al*., 1999), increased oxygen radical production (Sarkela *et al*., 2001), and nitration of critical biomolecules (Aulak *et al*., 2001; Elfering *et al*., 2004; Traaseth *et al*., 2004).

Several lines of evidence indicated that the gaseous molecule nitric oxide by binding to the heme moiety of soluble guanylate cyclase leads to its activation, and the formation of cGMP triggers a variety of events in various organs (Dawson *et al*., 1992; Garthwaite and Garthwaite, 1987; Marletta, 1989; Moncada *et al*., 1991). However, our research and that of others had indicated cytochrome oxidase as a different target for nitric oxide, by which mediates other processes not mediated or triggered by cGMP. Our hypothesis is that nitric oxide produced by mitochondria has a short-term regulatory role on energy metabolism, oxygen consumption, and the inherent free radical production. The broader implications of the present work can help to redefine the way we view regulation of oxygen consumption in vivo. On the basis of our initial findings, it has been proposed that mitochondrial production of nitric oxide helps average oxygen utilization between cells at different distances from capillaries. The basic concept is that nitric oxide will slow oxygen consumption by cells closest to blood vessels, allowing oxygen to penetrate to cells at the boundary of becoming hypoxic. In addition, nitric oxide would help dilate blood vessels and potentially increase oxygen delivery to borderline hypoxic cells (Giulivi, 2003; Haynes *et al*., 2003). Indeed, by following a similar line of thought, a mechanism for fireflight flashing has been proposed in which the role of nitric oxide is to transiently inhibit mitochondrial

respiration in photocytes and thereby increasing the availability and level of oxygen in the peroxisomes, oxygen being the species considered as the biochemical trigger for light production (Trimmer *et al*., 2001).

This emerging field in mtNOS is important, as it will expand the mechanisms by which cells consume oxygen and how changes in pO_2 are coped on a short-time framework. Studies in this field will provide key information on the molecular mechanisms of cellular respiration, and will likely lead to the design of better therapies to prevent pathological ischemic events during such diseases such as heart or brain stroke, and to advance our knowledge in the field of mitochondrial diseases.

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