MITOCHONDRIAL NITRIC OXIDE SYNTHASE IS CONSTITUTIVELY ACTIVE AND IS FUNCTIONALLY UPREGULATED IN HYPOXIA

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Abstract—Nitric oxide is a potent modulator of mitochondrial respiration, ATP synthesis, and K_{ATP} channel activity. Recent studies show the presence of a potentially new isoform of the nitric oxide synthase (NOS) enzyme in mitochondria, although doubts have emerged regarding the physiological relevance of mitochondrial NOS (mtNOS). The aim of the present study were to: (i) examine the existence and distribution of mtNOS in mouse tissues using three independent methods, (ii) characterize the cross-reaction of mtNOS with antibodies against the known isoforms of NOS, and (iii) investigate the effect of hypoxia on mtNOS activity. Nitric oxide synthase activity was measured in isolated brain and liver mitochondria using the arginine to citrulline conversion assay. Mitochondrial NOS activity in the brain was significantly higher than in the liver. The calmodulin inhibitor calmidazolium completely inhibited mtNOS activity. In animals previously subjected to hypoxia, mtNOS activity was significantly higher than in the normoxic controls. Antibodies against the endothelial (eNOS), but not the neuronal or inducible isoform of NOS, showed positive immunoblotting. Immunogold labeling of eNOS located the enzyme in the matrix and the inner membrane using electron microscopy. We conclude that mtNOS is a constitutively active eNOS-like isoform and is involved in altered mitochondrial regulation during hypoxia. © 2001 Elsevier Science Inc.

Keywords—Free radicals, Nitric oxide synthase, Mitochondria, Hypoxia

INTRODUCTION

Nitric oxide (NO) is a unique biological messenger molecule with various effects in cells and cellular organelles such as mitochondria. NO specifically interacts with mitochondrial function in different ways: it can compete with oxygen for cytochrome oxidase and so acts as an inhibitor of mitochondrial respiration [1–6]. Higher doses of NO also interact with complexes II and III to irreversibly block respiration and ATP synthesis [3,5,7]. Furthermore, NO stochiometrically reacts with superoxide to form peroxynitrite, which in turn activates protein kinase C (PKC), opens mitochondrial K_{ATP} channels, and, in higher doses, initiates cytochrome c release [7–10]. Therefore, mitochondrial NO synthesis could provide a powerful tool of self-regulation of the organelle and may modulate numerous physiological and pathophysiological reactions.

Three isoforms of NO-synthase enzymes are known to exist: the constitutive endothelial (eNOS) and neuronal (nNOS) isoforms and the inducible isoform (iNOS). Both constitutive isoforms are Ca-calmodulin dependent while the inducible isoform’s activity is not regulated by Ca^{2+} concentration [8]. Recent studies show the presence of a potentially new isoform of the enzyme in mitochondria (mtNOS) [11–14]. Studies of Bates et al. showed the presence of eNOS-like immunoreactivity in mitochondria isolated from various rat tissues [11,12]. Mitochondrial NOS activity was measured with different protocols in rat liver mitochondria [13,14]. The enzyme was purified from rat liver to homogeneity and was characterized as being similar to iNOS in molecular weight, Km, and cofactors [15]. However, linking mtNOS to the known isoforms based on antigenity yielded divergent results depending on the species and regions...
investigated. Furthermore, other authors had difficulty reproducing some of these experiments [2] and doubts emerged regarding the physiological relevance of NOS in mitochondria [16].

The aims of the present study were to: (i) examine the existence and distribution of mtNOS in mouse tissues using three independent methods, (ii) characterize the cross-reaction of mtNOS with antibodies against the known isoforms of NOS, and (iii) investigate the effect of hypoxia on mtNOS activity.

MATERIALS AND METHODS

Mitochondria preparation

Pure isolated mitochondria preparations were collected using Sims’ discontinuous percoll gradient method [17,18]. This protocol offers sufficient yield with very low contamination from other organelles (1–4%) [18]. All procedures were approved by the Animal Care and Use Committee of Wake Forest University. Wild-type mice from the C57BL/6 strain (Jackson, Bar Harbor, ME) were overanesthetized with halothane and decapitated. The brain and liver were removed, weighed, finely chopped, and homogenized by 8 strokes of a Teflon/glass motorized tissue grinder (Wheaton, Millville, NJ, USA) in isolation buffer containing 12% percoll (Amersham Pharmacia, Uppsala, Sweden). Mitochondria were isolated in sucrose buffer containing 0.32 mol/l sucrose, 1 mmol/l EDTA, 10 mmol/l Tris-HCl, pH 7.4. The resulting suspension was centrifuged for 3 min at 3000 rpm, and the middle layer was saved and layered on top of a discontinuous percoll gradient (24/40%). The gradient was centrifuged for 5 min at 19,000 rpm and the third layer containing the purified mitochondria was collected. The preparation was washed in isolation buffer and centrifuged for 10 min at 14,000 rpm and the pellet was used for further investigations. All procedures were performed on ice.

The purity of the mitochondrial preparation was tested by two independent methods. First, electron microscopic observations showed very little contamination from broken mitochondria or lysosomes; a few intact cells were found in some preparations. Second, the purity of the preparations was assessed by western blotting. The endoplasmic reticulum marker calreticulin was present in the whole tissue preparations but was not detectable in the purified mitochondria. In contrast, the mitochondrial marker cytochrome oxidase was significantly enriched in the mitochondria preparations (data not shown).

A group of animals were subjected to hypoxia (8% O2) for 25 min in a warm (37°C) chamber 6 h before mitochondria preparation.

NOS-activity assay

NOS activity was evaluated by labeled citrulline generation as described [19,20]. The assay was carried out in duplicates, using 100 µl of isolated mitochondria (protein concentration 1–4 µg/µl). From brain mitochondria preparations, 2–3 animals’ pellets had to be pooled to reach a sufficient amount. The samples were incubated with 75 µl of 14C-labeled arginine in Tris buffer, 25 µl of cofactors, and incubated for 30 min at 32°C. The 14C universally labeled arginine (specific activity 331 mCi/mmol, New England Nuclear, Boston, MA, USA) was purified through a Dowex iX-8 (acetate form) column.
and diluted in buffer containing 66.7 mmol/l Tris, 2.7 mmol/l NADPH, and 3.33 mmol/l calcium chloride at pH 7.4. Cofactors were added at a final concentration of 2 μmol/l for FMN, 2 μmol/l for FAD, and 10 μmol/l for BH₄. Arginase activity was inhibited by ornithine at a dose of 1 mmol/l [21].

After 30 min incubation, the reaction was stopped by placing the reaction tubes in ice water and by adding 1 ml of stop buffer containing 30 mmol/l 2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES) and 3 mmol/l EDTA at pH 5.5 to each tube. Separation of arginine from citrulline was performed by ion-exchange chromatography with 0.5 ml of Dowex-50 W resin (sodium form, 50X8-400, Sigma Chemical Co., St. Louis, MO, USA). After washing the column with a further 1 ml of stop buffer, the eluent containing the ¹⁴C-citrulline was collected in 7 ml scintillation vials and 3 ml of scintillation fluid was added (Bio-Safe II, Research Product International Corp., Mount Prospect, IL, USA). The protein concentration of the original samples was measured by a Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) and the enzyme activity was expressed in pmols of citrulline per milligram of protein per minute. Calmodulin dependence was evaluated by the addition of calmidazolium at a concentration of 100 μmol/l. Background counts were measured parallel with each sample, in the absence of cofactors and in the presence of 100 μmol/l N-nitro-L-arginine methyl ester, and were subtracted from each measurement. Statistics were carried out using Student’s paired or unpaired t-test as appropriate; p < .05 was considered significant. Data are presented as mean ± SEM.

Western blot

Protein was extracted from mitochondria by the addition of boiling lysis buffer (containing 1% v/v 1 mol/l Tris and 1% w/v sodium dodecyl sulphate). The samples were sonicated, heated at 95°C for 5 min, and centrifuged for 20 min at 12,000 rpm at 4°C. The supernatant was used for immunoblotting. Protein concentration was measured by a Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) and the enzyme activity was expressed in pmols of citrulline per milligram of protein per minute.

Electron microscopy

Freshly isolated mitochondria samples were fixed for 3 h in 4% paraformaldehyde/0.05% glutaraldehyde in pH 7.4 phosphate buffer. The samples were embedded in LR-white, sectioned, and collected on uncoated nickel grids. Two percent bovine serum albumin and 1:30 dilution of normal goat serum (homologous serum to secondary antibody) was applied to block unspecific binding. The sections were incubated overnight with primary
mouse anti-eNOS antibody in a dilution of 1:100 (BD Transduction Laboratory) at 4°C. Immunogold labeling was achieved by a 4 h incubation with 10 nm gold labeled goat antimouse IgG secondary antibody. The specificity of the procedure was tested by the omission of the primary antibody from the procedure, which resulted in the elimination of gold particles on the sections. Post-sectioning, en-bloc staining, and osmium postfixation were omitted to avoid interference with the labeling.

RESULTS

NOS activity

Nitric oxide synthase activity was measured in isolated brain and liver mitochondria and the respective tissue homogenates served as positive controls. NOS activity in the brain was significantly higher than in the liver (541 ± 53 pmol/mg/min, n = 5, vs. 17 ± 13 pmol/mg/min, n = 4, p < .01, respectively). Mitochondrial NOS activity was lower, but showed a similar difference between the two regions (liver: 9 ± 2 pmol/mg/min, n = 14, brain: 33 ± 3 pmol/mg/min, n = 5, p < .01). There was no significant difference between NOS activity measured in the liver or in the liver mitochondria. As a comparison, similar values for both whole tissue and mitochondria were obtained from samples of rat and porcine tissue (data not shown). Mouse liver preparations were chosen for further investigations, because this model offered better yield than the brain samples. The calmodulin inhibitor calmidazolium (100 μmol/l) completely inhibited mtNOS activity (Fig. 1A). Mitochondrial NOS activity from animals subjected to hypoxia was significantly higher than in the normoxic controls (Fig. 1B).

Western blot

In mouse liver mitochondria samples, immunoblotting with anti-eNOS antibody against the C-terminal 1025–1203 aminoacid sequence of the human enzyme showed a distinctive band at 140 kDa. Antibodies against the iNOS isoform (mouse C-terminal 961–1144 aminoacids) or the nNOS isoform (human 1095–1289 aminoacids) showed no visible band (not shown). In animals subjected to hypoxia, the mtNOS immunoreactive band was similar in intensity compared to the normoxic animals (Fig. 2).

Electronmicroscopy

The preparations contained mitochondria with well-preserved morphology and clearly defined cristae. Antibodies against the C-terminal fragment of eNOS were labeled with 10 nm gold particles. The majority of the mitochondria (> 90%) contained several gold beads located in the matrix and the inner membrane (Fig. 3). Heavily labeled nonmitochondrial particles were not observed in the sections. Omitting the primary antibody from the procedure resulted in elimination of the gold labeling.

DISCUSSION

In purified mitochondria preparations, we used three independent methods to document the presence of NOS in mitochondria. This is the first study providing direct evidence for the enzyme’s dependency on calmodulin and its functional upregulation following hypoxia.

Measuring NOS activity in mitochondria preparations from three species and two different organs showed comparable results. This finding suggests that a low level of NOS activity is a constitutive feature in mitochondria in general. The ability of NO production by mitochondria of single-cell organisms [22] indicates that this feature is an evolutionally preserved self-regulatory mechanism of the organelle.

In the present study, mtNOS showed cross-reactivity with eNOS but not with iNOS or nNOS, the enzyme was completely dependent on calmodulin, and its molecular weight was ≈ 140 kDa. Based on these data, it can be assumed that mouse liver mitochondria contain a constitutive, eNOS-like isoform of the enzyme. In rat liver mitochondria preparations, conflicting results appeared: Bates et al. found eNOS-like immunoreactivity, while Tatoyan and Giulivi reported the ineffectiveness of anti-eNOS antibodies [11,15]. In contrast, they found macrophage-NOS-like immunoreactivity, which represents the iNOS isoform. However, the constitutive expression of the enzyme and its complete dependence on Ca-calmodulin makes it less likely to be the inducible isoform [9,14,23,24]. It must be noted that most previous studies were conducted on rat liver preparations, and there may be a significant variation of the NOS isoforms present in mitochondria among different species and regions. Another possibility is that the enzyme, which is directed to the mitochondria, undergoes significant post-translational modifications altering its antigenity. All antibodies in the present study and in the literature, which reacted with mtNOS, were generated against the C-terminal of the respective enzyme [11,15,16,25], while the greatest divergence between eNOS and iNOS is at the calmodulin-binding domain. It is very likely that under different experimental conditions (i.e., electron microscopy or western blotting) a not ideally matching antibody reacts differently.

The most interesting finding in this study is the functional upregulation of mtNOS after hypoxia. It is well
described in the literature that NO affects mitochondrial function during hypoxia [4,9,26], though this study provides the first evidence for mitochondrial self-regulation by this system. Under conditions with restricted oxygen supply, the respiratory chain produces increased superoxide, which is suspected to be the main cause of hypoxic cell damage. Nitric oxide can substantially inhibit mitochondrial respiration and therefore superoxide production; on the other hand, NO readily reacts with superoxide to form peroxynitrite [8,24]. This dual mechanism can play a significant role in the defense against hypoxic damage as described in vivo [27]. This fact is also reflected in the relative hypoxia-resistance of NO-producing cell types like endothelial cells or nNOS-containing neurons [8].

Activation of mitochondrial NOS is reported to modulate matrix pH and respiration, although doubts have emerged whether the low activity of the enzyme is a significant contribution to physiological NO levels in the mitochondria [16]. Although NO is a diffusible agent, it is also very reactive and the local concentration of available substrates can restrict its action to a very limited space. In the mitochondrial matrix, the iron-containing cytochrome-oxydase and the superoxide anion are both readily available targets of NO; therefore, it is feasible to hypothesize that the primary function of mitochondrial

Fig. 3. Electron micrographs of isolated mitochondria. (A) Stained section showing the purity of the preparation. Isolated mitochondria have preserved morphology and well-defined cristae. (B–D) Immunogold labeling (10 nm) of eNOS in unstained sections. The majority of the mitochondria contain several gold beads, mostly located in the matrix or near the inner membrane.
NO production is localized to the organelle. Since mtNOS is a Ca-calmodulin-dependent isofrom, Ca$^{2+}$ accumulation in the mitochondria during hypoxia activates the enzyme [24]. In the course of hypoxic challenge, the increased NO production delays irreversible damage by decreasing mitochondrial activity.

In the present study, mtNOS activity significantly increased after hypoxic insult without apparent increase in NOS protein quantity as measured by immunoblotting. The physiological activity of extra-mitochondrial eNOS is regulated by phosphorylation. The enzyme can be phosphorylated at Ser-1179 by the Akt protein kinase [28] or the AMP-activated protein kinase [29,30], resulting in about 2-fold increase in NO catalytic activity. During hypoxia, when ATP levels drop and AMP accumulates, the AMP-activated protein kinase increases eNOS activity by Ca-calmodulin-dependent phosphorylation [29,30]. Furthermore, activation of AMP-activated protein kinase facilitates protein transport into the mitochondria [31]. In the present study, a 2-fold increase of mtNOS activity was found after hypoxic insult, which can be a result of phosphorylation. In this regard, further studies are needed to determine the sequence of mtNOS and test its reaction with protein kinases.

Nitric oxide has recently been shown to open mitochondrial K$_{ATP}$ channels directly or via PKC activation by peroxynitrite [32]. Pharmacological opening of these channels imitates the preconditioning effect of short-term mild ischemic episodes, however, the endogenous preconditioning agent has not yet been discovered. Nitric oxide is a reasonable candidate since it has the ability to open ATP-dependent potassium channels and its concentration increases during hypoxia. In contrast, NO in large quantities is a major mediator of hypoxic-ischemic tissue damage through activation of necrosis or apoptosis [33]. This controversy can be resolved by the hypothesis that the mitochondrially derived low NO concentration acts on the spot as a preconditioning agent, while the higher NO level produced by the other isofroms (especially iNOS) is responsible for cellular damage. As long as the hypoxic insult activates mtNOS but not iNOS, the preconditioning effect takes place. A stronger insult results in high NO production, which overrides the contribution of mtNOS to the effective NO level within the mitochondrial matrix.

We conclude that mtNOS is a constitutively active isofrom of NOS and it is involved in the altered mitochondrial regulation during hypoxia.

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**ABBREVIATIONS**

eNOS—endothelial nitric oxide synthase
iNOS—inducible nitric oxide synthase
mtNOS—mitochondrial nitric oxide synthase
nNOS—neuronal nitric oxide synthase
NO—nitric oxide
NOS—nitric oxide synthase
PKC—protein kinase C