Lack of mitochondrial nitric oxide production in the mouse brain

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Abstract
Based on our initial finding that the nitric oxide (NO) sensitive fluorochrome diaminofluorescein (DAF) was localized to mitochondria in cultured primary neurons, we investigated whether brain mitochondria produce NO through a mitochondrial NO synthase (mtNOS) enzyme. Isolated brain mitochondria were loaded with DAF and subjected to flow cytometry analysis. Neither the application of NOS inhibitors nor the genetic disruption of either NOS gene diminished the DAF-fluorescence. However, peroxynitrite scavengers reduced the mitochondrial DAF fluorescence, indicating that the DAF signal is not specific to NO. Chemiluminescence detection in the head space gas and a Clark-type NO-sensitive electrode in the solution failed to detect NO release in brain mitochondria. NOS activity in mitochondria was only 1% of the whole brain NOS activity level, which may be attributed to extramitochondrial contamination. Extensive immunoblotting and immunoprecipitation experiments failed to show the presence of endothelial, neuronal, or inducible NOS in mouse brain mitochondria using a variety of primary antibodies. Arginine, calmodulin or 2,5-ADP affinity purification protocols successfully concentrated eNOS and nNOS from full brain tissue but failed to show any signal in mitochondria. We conclude that mouse brain mitochondria do not contain NOS isoforms, nor do they produce NO through a NOS-dependent mechanism.

Keywords: diaminofluorescein, FP15, L-NAME, mitochondrial nitric oxide synthase, mitochondrion, peroxynitrite.


Nitric oxide (NO) and peroxynitrite (ONOO⁻) are considered to be pathological factors in a range of neurological disorders such as Parkinson’s disease, Alzheimer’s disease, multiple sclerosis, stroke and amyotrophic lateral sclerosis (Brown 1997; Heales et al. 1999). The basic mechanisms by which these nitrogen radicals induce cellular damage involve the inhibition of the mitochondrial respiratory chain (Robb et al. 1999; Murray et al. 2003). Low NO concentrations effectively compete with oxygen at the binding site of cytochrome c oxidase, which may serve as a physiological regulatory mechanism (Brown 1999, 2000). Also, cytochrome c oxidase was shown to be the enzyme responsible for the elimination of NO in mitochondria-rich cells (Pearce et al. 2002). Another mitochondrial pathway of NO metabolism is its nonenzymatic reaction with superoxide to form ONOO⁻. This potent, labile oxidant can also inhibit mitochondrial respiration in an irreversible manner at complexes II and III and it is involved in the induction of cell necrosis and apoptosis (Liaudet et al. 2000; Valdez et al. 2000). Furthermore, ONOO⁻ can nitrate the tyrosine residues of mitochondrial proteins, including most elements of complex I (Murray et al. 2003). Because NO is involved in so many aspects of mitochondrial function, it is an intriguing hypothesis that mitochondria themselves are capable of NO production.

The existence of a distinct mitochondrial NO synthase (mtNOS) has been suggested in several papers (Bates et al. 2001). However, the evidence for the existence of mtNOS is still controversial. In the present study, we investigated whether brain mitochondria produce NO through a mtNOS enzyme.

Received December 18, 2003; revised manuscript received March 19, 2004; accepted March 31, 2004.

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Abbreviations used: DAF-2-DA, 4,5-diaminofluorescein diacetate; DAF-FM, 4-amino-5-methylamino-2',7'-difluorofluorescein; DIC, differential interference contrast optics; eNOS, endothelial nitric oxide synthase; Hb, hemoglobin; iNOS, inducible nitric oxide synthase; i-NAME, N-nitro-l-arginine methyl ester; l-NNMA, N-methyl-l-arginine; mtNOS, mitochondrial nitric oxide synthase; nNOS, neuronal nitric oxide synthase; NOS, nitric oxide synthase.
The majority of the studies investigating mitochondria in the context of NO production were conducted on rat or mouse liver preparations (Giulivi et al. 1998; Tatoyan and Giulivi 1998; Arnaiz et al. 1999; Ghafourifar and Richter 1999; Kanai et al. 2001; Lacza et al. 2001; Boveris et al. 2002; Elfering et al. 2002; Riobo et al. 2002; Giulivi 2003; Schild et al. 2003). Some of the initial findings, however, were not reproducible in other laboratories challenging the physiological relevance of mtNOS (Brown 1999; Lacza et al. 2003a). The respiration control ratio of the preparation was 4.94 ± 0.46 in the presence of glutamate and malate (5 mM each). The cortical piece from the fetuses were washed twice in Dulbecco’s modified Eagle’s medium supplemented with penicillin (Sigma, 100 U/mL) and streptomycin (Sigma, 100 µg/mL) then were incubated with dispase I (2 U/mL, Roche, Mannheim, Germany) for 35 min at 37°C. Cells were dissociated by two series of gentle trituration and plated onto poly-1-lysine coated coverslips for confocal microscopic analysis. Mitochondria were prepared as described previously (Lacza et al. 2001; Schild et al. 2003). In case this mechanism is also present in the brain, one may hypothesize that it comprises a major factor in the pathogenesis of neurodegenerative diseases.

So far there are only a few studies that have attempted to identify brain mtNOS and they resulted in contradicting data. In an early paper, Bates et al. (1995) described eNOS immunoreactivity in brain mitochondria. Later, Riobo and colleagues measured NO release from rat brain mitochondria and described developmental changes in mtNOS activity (Riobo et al. 2002). In contrast, Henrich et al. (2002) found NOS immunoreactivity only in the close proximity of the mitochondria, but not in the organelles themselves, questioning the existence of mtNOS in neural tissues. This observation is supported by a study of Rothe and colleagues in which they found NADPH diaphorase activity, a marker of NO production, associated with the outer membrane of the mitochondria but not in the cristae (Rothe et al. 1999). Direct measurements of mitochondrial NO production or attempts to purify mtNOS from the brain have not been published in any species or preparations. Therefore, the present study aimed to investigate the existence of mtNOS and mitochondrial NO production with several overlapping methods in the brain.

**Experimental procedures**

**Mitochondria preparation**

All procedures were approved by the Animal Care and Use Committee of Wake Forest University. Mitochondria were prepared from halothane anesthetized mouse brain using the discontinuous Percoll gradient method as described previously (Lacza et al. 2003b). The respiration control ratio of the preparation was 4.94 ± 0.46 in the presence of glutamate and malate (5 mM each). The following mouse strains were used in the experiments: wild type C57BL/6; eNOS KO B6.129P2-Nos3tm1UNC, iNOS KO B6.129P2-Nos2tm1Lau; control for the eNOS KO C57BL/6 000664; nNOS KO B6.129S4-Nos1tm1Plh; control for the nNOS KO C57BL/6 J (Jackson, Bar Harbor, ME, USA). The purity of the mitochondrial preparation was tested by two independent methods. First, electron microscopic observations showed very little contamination of the mitochondria preparation by broken mitochondria or lysosomes. 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 reduced to insignificant amounts in the purified mitochondria. In contrast, the mitochondrial marker cytochrome c oxidase was significantly enriched in the mitochondria preparations. Special care was taken to ensure the best possible mitochondria preparations and purity testing was applied regularly to maintain the comparability of the results.

**Cell culture**

Primary rat cortical neurons were cultured from E18 Wistar rat fetuses as described previously (Kis et al. 2003). The cortical pieces from the fetuses were washed twice in Dulbecco’s modified Eagle’s medium supplemented with penicillin (Sigma, 100 U/mL) and streptomycin (Sigma, 100 µg/mL) then were incubated with dispase I (2 U/mL, Roche, Mannheim, Germany) for 35 min at 37°C. Cells were dissociated by two series of gentle trituration and plated onto poly-1-lysine coated coverslips for confocal microscopic analysis. After cell attachment, the plating medium was replaced by Neurobasal medium (Gibco BRL) supplemented with B27 (Gibco BRL, 2%), l-glutamine (Sigma, 0.5 mM), β-mercaptoethanol (Gibco BRL, 55 µM) and potassium chloride (Sigma, 25 mM). Cultures were grown at 37°C in humidified atmosphere containing 5% CO2 in air, and the medium was changed on every third day. Cultures consisted of more than 98% of neurons verified by positive immunostaining for microtubule-associated protein-2 (Becton-Dickinson), and negative immunostaining for glial fibrillary acidic protein (Chemicon, Temecula, CA, USA). Experiments were performed in 7–9-day-old cultures, a time period during which neurons express functional NMDA, AMPA and kainate receptors.

**Fluorescent confocal microscopy and flow cytometry**

Freshly isolated mitochondria were dispersed in K+-buffer containing 125 mM KCl, 2 mM K2HPO4, 5 mM MgCl2, 10 mM HEPES, 10 µM EGTA at pH 7.0 and plated on poly-1-lysine coated coverslips. Mitochondria were energized by the addition of sodium malate (5 mM) and sodium glutamate (5 mM) and were visualized using a Zeiss scanning confocal microscope (Axiovert 100 M) with differential interference contrast optics (DIC) and a fluorescein/ rhodamine filter set (excitation: 488 nm, emission: 505–530 nm; excitation: 543 nm, emission: > 560 nm, respectively). The fluorophores used were MitoFluorRed (1 µM) and 4-amo4-5-methyl- amino-2’,7’-difluorofluorescein (DAF-FM, 7 µM, Molecular Probes, Eugene, OR, USA). Mitochondrial fluorescence was observed under control conditions and in the presence of ONOO− scavengers tetrakis 2-triethylene glycol monomethyl ether(pyridil porphyrin (2-T(PEG3)PyP) (FP15, 100 µM), Inotec Corp, Beverly, MA, USA) (Szabo et al. 2002; Lacza et al. 2003; Pacher et al. 2003) or 5, 10, 15, 20-tetrakis(4-sulfonatophenyl)porphyrinato iron(III) chloride (FeTPPS, 100 µM, Calbiochem, San Diego, CA, USA). Background fluorescence of the probes or the mitochondria alone was below detection limit. 4,5-diaminofluorescein diacetate (DAF-2-DA, 7 µM, Molecular Probes) was used in cell cultures (Lopez-Figueroa et al. 2000) and DAF-FM in isolated mitochondria, where the necessary
enzymes to cleave the diacetate from DAF-2-DA are not present. In organelle-free solutions both DAF-2 and DAF-FM showed similar sensitivity to various NO donors (not shown).

Flow-cytometry was performed on freshly isolated mitochondria in K+-buffer in a similar fashion to the confocal measurements. Forward-scatter, side-scatter and fluorescence (FL1-H) were recorded by a BD-FacsCalibur flow-cytometer from 100,000 events in each preparation. Mitochondria-free buffer and buffer containing Percoll were used as negative controls. Data were evaluated by the CELLQuestPRO software.

Chemiluminescence NO detection

NO levels in the mitochondrial samples of mouse brain tissue were determined using an NO-analyzer (NOA 280, SIEVERS, Boulder, USA) in conjunction with the computerized data analysis program NOAWIN as described before (Roychowdhury et al. 2001).

For analysis of free NO in the samples, the reaction vessel was filled with water. The NO was carried from the reaction vessel to the analysis chamber by a steady flow of N₂. Chemiluminescence that resulted from the reaction of ozone with NO was measured via a photomultiplier. The instrument was calibrated by injection of constant volumes of head space samples from a reaction mixture that generated stoichiometric NO standards from the reaction:

\[ 2\text{KNO}_2 + 2\text{KI} + 2\text{H}_2\text{SO}_4 \rightarrow 2\text{NO} + \text{I}_2 + 2\text{H}_2\text{O} + 2\text{K}_2\text{SO}_4 \]

The lowest detection limit was found to be 50 pmol NO generated in 2 mL reaction mixture. To avoid any contamination of air, the reaction vessel (2 mL total volume) was purged with N₂ for 10 min before the experiment.

For measuring free NO in the mitochondria, freshly isolated mitochondria were dispersed in buffer (composed of 125 mM KCl, 2 mM K₂HPO₄, 5 mM MgCl₂, 10 mM HEPES, 1 μM CaCl₂ at pH 7.0) in presence of malate/glutamate (5 mM each) and ADP (2 mM) at a protein concentration of 1 mg/mL. After 5 and 20 min the samples were transferred to an air-tight oxygen-free vial followed by measurement of head space NO.

To rule out the possibility that the lack of NOS cofactors is responsible for the lack of NO production we added such substances to the mitochondria suspension. For this purpose, K⁺ buffer (for composition see Fluorescent confocal microscopy and flow cytometry) was mixed with 100 μM L-arginine (Alexis Biochemicals, San Diego, CA, USA), 0.2 mM NADPH (Sigma, St. Louis, MO, USA), 10 μM tetrahydrobiopterin (THB; Alexis Biochemicals), 5 μM flavin adenine dinucleotide (FAD), 5 μM flavin adenine mononucleotide (FMN), 10 μg/mL calmodulin (Bio-Rad), and transferred to polyvinylidene difluoride membrane. After blocking with 5% milk, primary antibody was applied overnight followed by horseradish peroxidase conjugated secondary antibody. Chemiluminescence was used to visualize the bands.
USA); anti-nNOS 1095–1289 (monoclonal and polyclonal, Transduction Laboratories); anti-nNOS 251–270 (polyclonal, Sigma); anti-nNOS 1383–1398 (polyclonal, Transduction Laboratories); anti-nNOS 1–181 (monoclonal, Sigma); anti-nNOS 1409–1429 (polyclonal, Sigma); anti-nNOS polyclonal SC-648 (Santa Cruz Biotechnologies); anti-iNOS 961–1144 (monoclonal, Sigma); anti-iNOS 772–787 (monoclonal, Sigma).

Immunoprecipitation was performed on freshly isolated mitochondrial proteins both in native and denatured conditions. Equal amounts of protein were incubated with anti-nNOS polyclonal antibodies at 4°C overnight in a volume of 750 µL. The antibodies were precipitated by the addition of 40 µL protein A/G coupled to beaded agarose and centrifuged at 1200 g for 30 s. The precipitate was tested with western blotting as described above.

Affinity purification
The hypothetical mitochondrial nitric oxide synthase was purified using cofactors of NOS immobilized on beaded sepharose. Mitochondria and full tissue homogenates were lysed with the addition of 1% NP-40 (Sigma) to preserve the native forms of the proteins. The lysates were prepurified based on arginine binding by loading onto 1 mL arginine-sepharose columns (Sigma) and eluting with excess arginine. The eluates were further purified by NADPH binding using a 2,5-ADP-sepharose column (Sigma) or by calmodulin binding using a calmodulin-sepharose column (Sigma). The columns were washed and the bound proteins were denatured by adding Laemmli sample buffer (Bio-Rad) and heated to 95°C for 5 min. Unconjugated sepharose was used as a negative control. The purified proteins were probed with western blotting as described above.

Results
Fluorescent measurements
Mitochondrial NO production was assessed by using the NO-sensitive fluorescent dyes, DAF-FM or DAF-2-DA. These compounds have a weak green fluorescence, which is greatly increased in the presence of NO. However, previous studies have shown that the increase of the dye’s fluorescence was much greater when the NO donors were applied together with ONOO⁻. Therefore, we used DAF fluorescence as an indicator of nitrogen radical production, that includes both NO and ONOO⁻.

Mitochondrial nitrogen radical production was investigated in cultured primary neurons with laser scanning confocal microscopy. The cells were loaded with MitoTrackerRed to label mitochondria and with DAF-2-DA to monitor nitrogen radical production. The DAF-2 fluorescence was completely colocalized with the mitochondria, although not every mitochondrion was labeled with DAF-2 (Fig. 1). Interestingly, the mitochondrial fluorescent signal increased when the preparations were exposed to the scanning laser light for a long time. This observation reflects that the main sources of nitrogen radicals are the mitochondria. The subsequent experiments were conducted on isolated brain mitochondria preparations.

Freshly isolated respiring mitochondria were visualized by confocal microscopy and the quantification of the fluorescence was achieved by flow cytometry in separate experiments. The mitochondria showed very strong green fluorescence in the presence of DAF-FM while the autofluorescence of the mitochondria was below the detection limit. DAF-FM fluorescence was observed in all three NOS knockout strains with confocal microscopy and it was not significantly different when quantified by flow-cytometry (Fig. 2). The DAF-FM fluorescence in the isolated mitochondria was unaffected by the application of the pharmacological NOS inhibitors like L-NAME or L-NMMA (up to 1 mM) (Fig. 3). In contrast, the mitochondrial nitrogen radical signal was significantly blocked by the application of the ONOO⁻ decomposition catalysts FP15 or FeTPPS (Fig. 3). Prolonged treatment of the animals with 1 mg/mL L-NAME for 3 days in the drinking water and supplementing L-NAME in the preparation buffers also failed to attenuate the DAF-FM signal. Moreover, the signal was insensitive to Ca²⁺ or to the withdrawal of the substrate L-arginine (Fig. 3).

Chemiluminescent NO detection
The results with DAF fluorescence showed prominent mitochondrial nitrogen radical production. However, this
method cannot differentiate between NO, ONOO⁻, or other NO-derived reactive species. Therefore, we attempted to measure genuine NO concentration with the SIEVERS chemiluminescence NO Analyzer in samples from the headspace gas of isolated respiring brain mitochondria. This methodology is able to detect 50 pmol NO in a 2 mL reaction vessel (as calibrated with NO donors, see Table 1), which is significantly lower than the lowest physiologically relevant NO concentration. All samples drawn from the headspace of the mitochondria suspensions at any of the investigated conditions showed no detectable NO levels even in the presence of exogenous l-arginine (Table 1). Also the addition of cofactors of the NOS enzyme did not result in detectable NO-levels.

Electrochemical NO detection
A previous study has shown NO production by isolated heart mitochondria with an electrochemical electrode. This method allows the continuous measurement of NO in the solution and genuine NO can be confirmed by the addition of hemoglobin in the end of each experiment. In our hands, this method also failed to detect any signal, which was attributable to NO in the brain mitochondria preparations (Fig. 4).

NOS activity assay
Isolated brain mitochondria had a low NOS activity, which amounted to less than 1% of the full brain NOS activity (Fig. 5). Such low mitochondrial NOS activity was partially inhibited by either L-NAME or the calmodulin inhibitor calmidazolium (Fig. 5). Mitochondria isolated from eNOS, iNOS and nNOS knockout animals had a comparable activity as the respective wild-type strains (Fig. 5). As a comparison, NOS activity was also measured in full brain tissue homogenates from the same animals as used for the mitochondria preparations. The measured full tissue activity was comparable in all strains, and it was greatly reduced in nNOS knockouts (Fig. 5).

Western blotting and immunoprecipitation
Previous studies have demonstrated immunoreactive bands in mitochondria preparations when labeled with specific NOS antibodies, while other studies reported negative results using the same antibodies (for a review see Lacza et al. 2003a). We have chosen to screen the preparations from brain mitochondria with a set of 10 different NOS antibodies to test the antigenity of mtNOS. To identify the respective eNOS, nNOS or iNOS bands we used known positive controls obtained from tissues of wild-type as well as NOS knockout animals.

Mitochondria from normal mouse brain tissue did not show any immunoreactive proteins tested with two different anti-iNOS antibodies, an anti-eNOS antibody or a universal NOS antibody (Fig. 6). However, there were other mitochondrially enriched bands when probed with several anti-nNOS antibodies. These bands had a lower molecular weight (70–110 kDa) than expected for nNOS isoforms and were also present in the nNOS KO animals (Fig. 7). Because nNOS is the only NOS with more than one known protein splice variants and the exon2 knockout used in the present study...
and other studies lacks only the most abundant nNOSα isoform, the possibility remained open that these other bands represent a mitochondrial splice variant of nNOS. In order to test this hypothesis we screened the mitochondria with six different anti-nNOS antibodies. None of these lower molecular weight bands reacted with more than one antibody, making it unlikely that these proteins share significant homology with nNOS.

In order to reduce non-specific binding to the antibodies, next we used immunoprecipitation protocols. Two polyclonal antibodies, one against the oxygenase domain and one against the reductase domain, were used to precipitate nNOS in separate experiments. The isolated proteins were probed with western blotting using other anti-nNOS antibodies raised against different epitopes. This approach resulted in the concentration of nNOSα and nNOSβ in full brain tissue (Fig. 7). However, no mitochondrial proteins were found (Fig. 7), not even the secondary bands, which were seen with western blotting (see above), making it very unlikely that these lower molecular weight bands represent unknown splice variants of nNOS.

Affinity purification

Brain mitochondria and full brain tissue samples were loaded onto arginine-sepharose columns and eluted with excess arginine. The eluent from the full tissue, but not from the mitochondria contained nNOS as shown with western blotting (Fig. 7). Subsequent affinity purification with the NADPH analog 2,5-ADP resulted in a further concentration of nNOS in the brain, still without any nNOS-like protein in the mitochondria (Fig. 7). Similar results were obtained with calmodulin-sepharose (Fig. 7). Probing of the affinity purified proteins with an anti-eNOS antibody also resulted in a strong band in the full tissue samples with no mitochondrial proteins at all. Probing with an anti-iNOS antibody did not show any immunoreactive bands in the preparations.

Discussion

There are two main observations in the present study. First, we showed that brain mitochondria do not produce NO via the arginine-to-citrulline conversion pathway. Second, we did not find significant levels of NOS enzymes in the mitochondria. Both of these observations indicate that mtNOS is not present in brain mitochondria.
Based on the growing literature of mtNOS and our previous experiments with liver mitochondria, we expected to see significant mitochondrial NO production in the brain. First, we visualized the mitochondria in cultured neurons and found that the NO-sensitive DAF fluorescence colocalizes with the mitochondria. This observation was confirmed in isolated mitochondria and was quantified by flow cytometry in the present study. Several other groups have published similar results in various preparations indicating that the localization of DAF fluorescence to the mitochondrion is a general observation (Lopez-Figueroa et al. 2000; Dennis and Bennett 2003). However, withdrawal of arginine and Ca\(^2+\) from the solution failed to decrease mitochondrial DAF fluorescence. The signal was not inhibited by the application of classical, competitive NOS inhibitors like L-NAME or L-NMMA, even in high doses (1 mM) or prolonged application (3 days pretreatment before mitochondria preparation). Furthermore, the DAF fluorescence was similar in mitochondria prepared from all three NOS knockout strains. These observations reflect that the mitochondrial conversion of DAF to a fluorescent product is not the result of a classical NOS enzyme activity. Our experiments with ONOO\(^–\) decomposition catalysts suggest that other nitrogen radicals besides NO may be responsible for mitochondrial DAF fluorescence. Furthermore, DAF staining of the mitochondria was very heterogeneous both in cultured cells and in isolated organelles. Further studies are needed to understand the chemistry of DAF before we can form a conclusion of the nature of the mitochondrial DAF fluorescence. One possible working hypothesis is that mitochondria generate NO from nitrite via a mechanism that does not involve the NO synthases (Nohl et al. 2000), followed by a rapid reaction of NO with superoxide (produced by the mitochondrial oxidative processes). Another possibility is that the DAF fluorescence, in mitochondria, is related to some peculiarity of the probe, when reacting with reactive oxygen species, which can also be neutralized by the porphyrinic antioxidants used in our studies.

Since the specificity of DAF to NO has been questioned in other preparations also, we tried to detect mitochondrial NO
formation by direct methods (Jourd’heuil 2002; Roychowdhury et al. 2002; Zhang et al. 2002). Both the chemiluminescence and the amperometric detection are sensitive methods to measure NO release from mitochondria as demonstrated in liver tissue (Schild et al. 2003). Under these well-controlled situations isolated respiring brain mitochondria did not produce detectable amounts of NO either in the solution or in the headspace gas. We altered the respiratory status or supplemented the availability of substrate or potential cofactors but still no NO production was observed. These observations are in contrast to several studies in the literature. Riobo and colleagues used hemoglobin oxidation to indirectly measure NO production in brain mitochondrial membranes (Riobo et al. 2002). In the present study, we used a direct NO detection method and we did not observe any changes, which would be inhibitable by hemoglobin in brain mitochondria. One explanation of these divergent data can be that mtNOS is only active at the early stages in brain development, and the adult mtNOS levels are just insignificant remnants of the embryonic stage, with some variations among species and animal strains.

If there is no detectable NO production in the mitochondria, why do we measure arginine conversion, although at very modest levels? The arginine to citrulline conversion assay optimizes the conditions for the NOS enzymes: the substrate and the cofactors are abundant and other arginine-metabolizing enzymes are inhibited. Under these conditions, we were able to detect a very weak mitochondrial NOS activity signal, which amounted to less than 1% of the full tissue activity. As even the best mitochondria preparations contain 1–4% non-mitochondrial membranes (Sims 1990), it is impossible to distinguish whether this very low NOS activity belongs to the mitochondria or the contaminant membranes. It is also possible that arginine conversion is the result of other enzymes like arginase II or arginine decarboxylase, both of which are abundant mitochondrial enzymes. Therefore, the mitochondrial NOS activity measurements in the present study cannot prove the existence of a distinct mtNOS enzyme.

Extensive screening of the mitochondrial proteins using a set of 11 NOS antibodies failed to show the presence of any specific NOSs in brain mitochondria. We used full brain homogenates as positive controls and the respective NOS knockout strains as negative controls. Immunoprecipitation protocols were applied to concentrate nNOS in the mitochondria. This procedure successfully eliminated the non-specific bands and concentrated the known nNOS splice variants (a, b, c) in the brain, but not in mitochondria. We also tried to purify mtNOS based on its affinity to the substrate or cofactors of NOS. This procedure very effectively concentrated eNOS and nNOS in full brain tissue, however, mitochondria preparations did not contain any NOS immunoreactive proteins even after affinity purification. Furthermore, we also tried to find NOS immunoreactivity in the mitochondria with immunogold electron microscopy, without any success (Lacza et al., unpublished observations). This finding is also supported by the lack of mitochondrial transport tags in the sequences of either NOS protein, which we published recently (Lacza et al. 2003a).

![Western blot](image1.png)

**Fig. 7** Search for nNOS splice variants in mitochondria. Panel A shows a representative western blot with a monoclonal anti-nNOS antibody, which recognizes all known nNOS splice variants. Full brain tissue contains nNOS, which is missing in the nNOS KO animal. An approximately 75-kDa band is enriched in the mitochondria compared to the full brain. This band is similarly present in mitochondria from both control and nNOS KO mice, indicating that it cannot be a degradation product of nNOS. (b) shows the immunoprecipitation of brain and mitochondria preparations with a polyclonal anti-nNOS antibody, the precipitate was blotted with a different monoclonal antibody against the same epitope. Two nNOS splice variants (nNOS and nNOSb) were identified in the full brain, but no mitochondrial proteins were found. (c) shows the results of the affinity purification of nNOS from brain and brain mitochondria. Arginine (Arg), 2,5-ADP (ADP) or calmodulin (Cam) affinity purification effectively concentrates the nNOS form the brain tissue, however, no mitochondrial bands were found.
One possible explanation for the divergent data in the context of mtNOS is that cellular NOS is attached to the mitochondria in a similar manner like eNOS is localized to the caveolae in the endothelial cell membrane. Indeed, several studies described NOS immunoreactivity or NADPH diaphorase activity in the proximity of the mitochondria, but not in the organelles themselves (Frandsen et al. 1996; Rothe et al. 1999; Henrich et al. 2002). The present study shows that mitochondria are not capable of NO production when they are separated from other cellular elements, nor they contain a mtNOS, however, there may be a functional attachment of NOS enzymes to the outer mitochondrial membrane in the intact cell. This phenomenon may account for the positive results in some earlier studies (Bates et al. 1995; Rothe et al. 1999; Lacza et al. 2001). Further, our data undermine the hypothesis that mtNOS plays a role in the pathomechanism of neurological disorders.

Acknowledgements

The authors are grateful for Ken Grant for his invaluable help with confocal and electron microscopy and Dr Bela Kis for culturing neurons. We also thank Dr Martha Alexander-Miller for her suggestions on flow-cytometry. This study was supported by grants from the American Heart Association (Mid-Atlantic Grant 99512724, Bugher Foundation Award 0270114 N) and the NIH (HL30260, HL46558, HL50587, HD38964); the Hungarian OTKA 99512724, Bugher Foundation Award 0270114 N) and the NIH from the American Heart Association (Mid-Atlantic Grant

References


Mitochondria do not produce nitric oxide


