Nitric oxide synthase activity in mitochondria

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Abstract In the present study we show the existence of a functional nitric oxide synthase (NOS) in rat liver mitochondria. The enzyme uses L-arginine (L-arg) to produce nitric oxide (NO) and L-citrulline, and is Ca^{2+}-dependent. L-Arg analogues, N^ω-monomethyl-L-arg and N^ω-nitro-L-arg, inhibit the enzyme, and D-arginine is not a substrate for it. We found mitochondrial NOS (mtNOS) activity associated with the inner mitochondrial membrane but not with the matrix fraction. In intact, succinate-energized mitochondria, the enzyme is constitutively active and exerts substantial control over mitochondrial respiration and membrane potential. The activity is further stimulated when Ca^{2+} is taken up by mitochondria. We suggest that the existence of mtNOS and its Ca^{2+} dependence are highly relevant for mitochondrial functioning.

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Key words: Nitric oxide; Mitochondrial respiration; Mitochondrial membrane potential

1. Introduction

Nitric oxide (NO, nitrogen monoxide) is a widespread intra- and intercellular messenger with a broad spectrum of activities in the central nervous, cardiovascular, and immune systems [1]. The biosynthesis of NO is accomplished by oxidation of a terminal guanidino nitrogen of arginine, yielding citrulline as a coproduct, and is catalyzed by members of the NO synthase (NOS) family [2]. Three distinct isoforms of NOS have been identified in mammalian tissues, referred to as endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) [3], although none of these enzymes has an absolutely tissue-specific pattern of expression. The isoforms are present in different locations within the cells: nNOS and iNOS are cytosolic [4], whereas eNOS has been shown to be membrane-associated [5–8].

Stimulation of soluble guanylyl cyclase accounts for important biological responses to NO [1]. Recently it has become evident that also mitochondrial activities are strongly affected by NO [9–21]. NO is able to bind to cytochrome oxidase and thereby to act as an inhibitor of mitochondrial respiration [9,11,12,14–20]. As a consequence of cytochrome oxidase inhibition, Ca^{2+} is released from mitochondria [9,10,21] in parallel to their de-energization [9]. The existence of a Ca^{2+}-dependent mitochondrially located NOS might, therefore, have important biological implications.

Since 1995 contradictory results concerning the possible presence of NOS in mitochondria have been published. Immunohistochemical studies, using NADPH diaphorase staining [22,23] and silver-enhanced gold immunolabelling [24], provided evidence for the presence of NOS-like proteins in several locations within the mitochondria. Co-localization of eNOS with succinate dehydrogenase as a mitochondrial marker [14] and cross-reaction of mitochondria with antibodies directed against eNOS [22,24] have been reported. Although it was clearly shown that eNOS is primarily localized in membranes, previous studies [22,24] have shown eNOS staining in several locations, including the membranes, within mitochondria. Cross-reaction of mitochondria with nNOS antibodies has also been reported [23]. One study [14] reported faint NOS activity in a preparation of rat diaphragm muscle mitochondria, but did not rule out the presence of non-mitochondrial NOS, nor the influence of the urea cycle in citrulline formation, which had been used as the indicator of NOS activity.

In the present study we demonstrate, using established methods, (i) NOS activity in mitochondria, where it is located at the inner membrane, (ii) that mitochondrial NOS (mtNOS) activity is substantial and modulated by Ca^{2+}, and (iii) that mtNOS continuously controls mitochondrial respiration.

2. Materials and methods

2.1. Chemicals

L-[2,3-^{3}H]Arginine (36.8 Ci/mmol) was obtained from Sigma, L-[ureido-^{14}C]citrulline (58.8 mCi/mmol) from New England Nuclear. Oxyhemoglobin (HbO$_2$) was prepared as described by Di Iorio [25]. Human recombinant calmodulin was a generous gift of Dr. D. Guerini, Zurich, Switzerland. Recombinant rat brain nNOS was kindly provided by Dr. B. Mayer, Graz, Austria. All other chemicals were purchased from standard suppliers, and were of the highest purity commercially available. Chemicals were prepared fresh.

2.2. Isolation of mitochondria and their subfractions

Tightly coupled liver mitochondria were prepared by differential centrifugation as described [26]. The protein content was determined by the biuret method with bovine serum albumin as standard. Broken mitochondria (BM) were prepared from intact mitochondria (IM) (60–100 mg/ml) as follows. After a hypoosmolar shock in four times the volume of distilled water the mitochondrial suspension was sonicated (Sonifier, Branson Sonic Power Co., operated at 150 W and 50% duty cycle) for 75 s. The protein content was again determined and adjusted with buffer to 20–30 mg/ml. The completeness of membrane disruption was verified by the absence of a mitochondrial membrane potential ($\Delta\Psi$) and Ca^{2+} uptake [27]. To obtain mitoplasts (MP), mitochondria were treated with 0.12 mg digitonin/mg mitochondrial protein for 15 min at 4°C and washed twice [28]. Submitochondrial particles (SMP) were prepared from MP after hypoosmolar shock and sonication, as described for BM preparation, followed by centrifugation at $9500 \times g$ for 10 min [26]. The pellet is referred to as SMP. The supernatant was then centrifuged at $100\,000 \times g$ for 35 min

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Abbreviations: BM, broken mitochondria; $\Delta\Psi$, mitochondrial membrane potential; IM, intact mitochondria; L-NMMA, N^ω-monomethyl-L-arginine; metHb, methemoglobin; mtNOS, mitochondrial nitric oxide synthase; MP, mitoplast; NO, nitric oxide (synonym nitrogen monoxide); L-NA, N^ω-nitro-L-arginine; NOS, nitric oxide synthase; HbO$_2$, oxyhemoglobin; SMP, submitochondrial particles; SOD, superoxide dismutase

at 4°C. The supernatant of this ultracentrifugation is referred to as matrix fraction.

2.3. Spectrophotometric determination of NO

The measurement of NO is based on its reaction with HbO_2 and the subsequent formation of methemoglobin (metHb), as described [29]. Measurements were done at 37°C with a Varian CARY spectrophotometer at 401 nm. MetHb formation was recorded at various times and quantified by calculations based on an extinction coefficient of 49 mM^{-1} cm^{-1} for metHb [29]. Addition of mitochondria or mitochondrial subfractions (0.03 mg/ml) was taken as time 0. The medium contained HbO_2 (4 µM) and NOS substrates as follows: L-arg (1 mM), $CaCl_2$ (1 mM), NADPH (0.2 mM), FAD (5 µM), FMN (5 µM), and BH_4 (10 µM) in HEPES buffer (0.1 M, pH 7.4). NO can rapidly react with superoxide radicals to produce peroxynitrite. Since mitochondria are a well known source of oxygen radicals [30], and since high activity of mtNOS reported here may lead to substrate and cofactor limitation, which in turn will cause simultaneous generation of superoxide and NO by NOS [31–33], 1 kU/ml of superoxide dismutase (SOD) [31] was also provided in this assay. The blank cuvette contained buffer, HbO_2, NOS substrates and SOD. NO formation was tested under the same condition using 3 µg of recombinant rat brain NOS, NOS substrates, and 10 µg/ml of calmodulin in the presence or absence of BM and IM.

2.4. Citrulline measurement

Measurements were done as described [34] except for modifications in the size and shape of the cation exchange columns. In a final volume of 0.1 ml mitochondria or mitochondrial subfractions were incubated as follows. For the protein concentration dependence experiments, 0.01–1 mg of mitochondria or mitochondrial subfractions were added to the buffer (37°C) containing NOS substrates supplemented with L-[³H]arg (30 000–50 000 cpm), and incubated for 2 min. For the time dependence, 1 mg of IM or SMP was added to buffer (final volume 0.1 ml, 37°C) containing NOS substrates supplemented with L-[³H]arg (30 000–50 000 cpm), and incubated for various times. For the investigation of basal mtNOS activity, mitochondria were added to the buffer supplemented only with L-[³H]arg (30 000–50 000 cpm) (without NOS substrates), incubated for 2 min, and then energized with 0.8 mM K^+-succinate. For studying the Ca^{2+} dependence of mtNOS, mitochondria were added to the buffer containing 0.8 mM K^+-succinate and NOS substrates supplemented with L-[³H]arg (30 000–50 000 cpm) as above, except for using varying Ca^{2+} concentrations.

The reaction was terminated by addition of 1 ml of chilled 20 mM sodium acetate buffer (pH 5.0) containing 2 mM EDTA and 1 mM unlabeled L-citrulline. For separation of L-[³H]citrulline from L-[³H]arg, half of the final volume (550 µl) was put on spin columns (Spin Module, Size 100, Bio-101, Vista, CA) filled with 0.3–0.4 ml of the cation exchange resin (Dowex 50W, 8% cross-linkage, 200–400 mesh size, Na^+ form) [34]. Loaded columns were centrifuged at $7000 \times g$ for 2 min and washed with 200 µl of buffer. The total effluent was analyzed by liquid scintillation counting. The columns and the resin were tested with both L-[³H]arg and L-[ureido-^{14}C]citrulline for their efficiency.

2.5. Oxygen consumption measurement

Oxygen consumption of K^+-succinate-energized IM, BM, MP or SMP (1 mg protein each) was measured at room temperature with continuous stirring in 1.3 ml buffer with a Clarke-type electrode, as described [35]. In Fig. 3A–C, mtNOS stimulation or inhibition was achieved by the addition of mitochondria or mitochondrial subfractions to the chamber contained NOS substrates (as mentioned above) and/or the inhibitor, and then energized with 2.5 mM K^+-succinate (arrow). In Fig. 3D, addition of NOS substrates is indicated by the triangle.

2.6. Determination of mitochondrial membrane potential

$\Delta\Psi$ of succinate-energized mitochondria (1 mg/ml) was measured in an Aminco DW-2A spectrophotometer at 511–533 nm in the presence of 10 µM safranin as described [27]. At the end of the measurement, the uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (1 µM), was added to ascertain the validity of the measurement.

2.7. NOS inhibition

N^{ω}-monomethyl-L-arg (L-NMMA) (1–3 mM) and N^{ω}-nitro-L-arg (L-NA) (1–5 mM) were used for NOS inhibition. In the spectrophotometric assay, the inhibitor was added before NOS substrates. In the citrulline and O_2 consumption measurements, mitochondria or mitochondrial subfractions were incubated with the inhibitors for 2–5 min prior to the addition of NOS substrates. Incubation with the solvent alone served as control. In $\Delta\Psi$ measurements, the inhibitor was added prior to mitochondria.

3. Results

NO is highly unstable in aerobic aqueous solutions. It can be quantitatively measured via its reaction with HbO_2 to form metHb, which can be detected as an increase in the optical density at 401 nm [29]. As shown in Fig. 1, the production of NO measured by this technique was detected after the addition of BM to NOS substrates. Fig. 1 also shows that 1 mM L-NMMA abrogates NO formation. Complete inhibition was also achieved with 5 mM L-NA ($n = 4$). When IM or MP were used instead of BM, no NO formation was detectable with this assay, ruling out a contamination of our mitochondrial preparation with non-mitochondrial NOS. The HbO_2 assay is not totally specific for NO, but the presence of relatively high amounts of SOD in this assay prevents the possible interaction of HbO_2 with other reactive oxygen or nitrogen species, e.g. superoxide or peroxynitrite. Addition of 3 µg of recombinant rat brain nNOS to the buffer containing NOS substrates in the presence or absence of BM or IM resulted in a time-dependent NO formation (not shown) which was inhibited by L-NMMA (3 mM) or EGTA (5 mM), but not by the arginase inhibitor, L-lysine [36] (1 mM).

To investigate the location of NOS in mitochondria, both SMP and the matrix fraction were also tested. Fig. 1 shows the formation of NO by SMP, which is completely inhibited by 1 mM L-NMMA. The same full inhibition was also achieved by 3 mM L-NA (not shown). The matrix fraction did not show any NOS activity ($n = 6$).

In the above-mentioned experiments with BM and SMP, omission of Ca^{2+} ($n = 8$), NADPH ($n = 4$), BH_4 ($n = 4$) or FMN ($n = 4$), or replacement of L-arg with its stereo-isomer, D-arginine ($n = 4$), reduced NO formation (10–50% and 5–30% of control for BM and SMP, respectively), and addition of EGTA (5 mM, $n = 4$) abrogated it. With less than 300 U SOD/ml NO was hardly detectable. Addition of calmodulin (10 µg/ml) did not increase metHb formation. In the presence of the
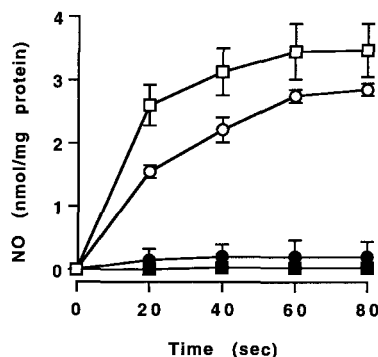

Fig. 1. Time-dependent NO formation in mitochondrial subfractions measured spectrophotometricaly. NO formation by BM (□) and SMP (○) was measured by following the conversion of HbO_2 to metHb at 401 nm. Inhibition by 1 mM L-NMMA of NO formation is shown for BM (■) and SMP (●). Each point represents the mean ± S.E.M. of eight independent experiments.

NO scavenger, carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide [37] (0.5 mM), complete inhibition ($n = 4$) of metHb formation was achieved.

Another widely used assay for NOS activity is the measurement of L-[³H]citrulline produced from L-[³H]arg. Fig. 2A shows that mtNOS activity thus assayed in IM, BM, and MP is protein concentration-dependent. Citrulline formation is sensitive to L-NA (Fig. 2A, inset) and to L-NMMA (not shown). The amount of citrulline produced in 1 min by 1 mg of BM (3.8 ± 0.76 nmol) (Fig. 2A) is close to the amount of NO measured with BM in the HbO_2 assay after 1 min (3.5 ± 0.44 nmol/mg) (see Fig. 1). Citrulline formation by both IM and SMP is time-dependent and L-NA-sensitive (Fig. 2B). Citrulline production was not inhibited by 1 mM L-lysine: 5.2 ± 0.76 and 8.7 ± 1.05 nmol citrulline/mg/min ($n = 3$) were formed by IM in the absence or presence of L-lysine, respectively. The sensitivity of citrulline formation towards L-NA and L-NMMA and its insensitivity towards L-lysine strongly suggest that the detected citrulline was due to NOS activity. Heat-treated (80°C, 5 min) IM, BM and MP did not form citrulline. No citrulline was formed by the matrix fraction.

Fig. 3. Effect of mtNOS on O_2 consumption by mitochondria and mitochondrial subfractions. Oxygen consumption was induced with 5 mM K^+-succinate (arrow) in 1 mg IM (A), 1 mg frozen/thawed (5 cycles) IM (B), and 1 mg SMP (C). Trace a, control O_2 consumption; trace b, as trace a, but after mtNOS stimulation as described in Section 2; trace c, as trace b, but in the presence of L-NMMA; trace d, O_2 consumption of mitochondria or mitochondrial subfractions in the presence of L-NMMA, without NOS substrates. D is as A, but mtNOS stimulation was achieved by addition of NOS substrates at the triangle (trace I). Trace II is as trace I, but Ca^{2+} was omitted from the NOS substrates. Traces are representative of four to six independent experiments.
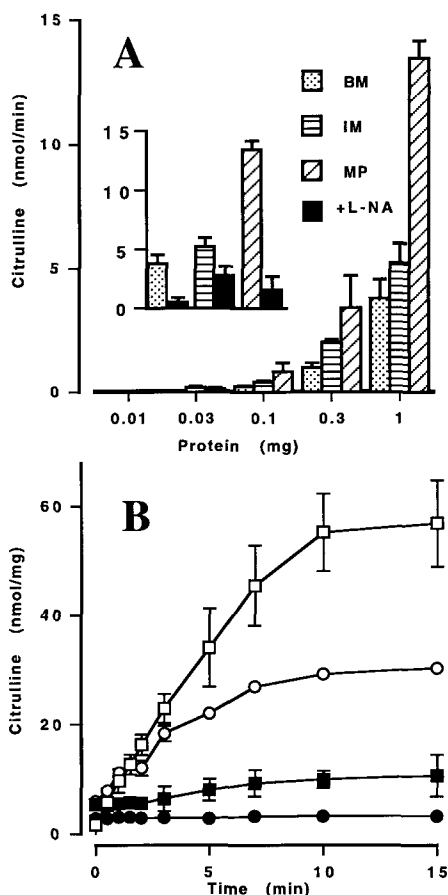

Fig. 2. Mitochondrial protein concentration and time dependence of mtNOS. A: Protein concentration-dependent activity of mtNOS in BM, IM, and MP determined by measuring the formation of L-[³H]citrulline. Inset: Inhibition by L-NA of citrulline formation by 1 mg of mitochondria or mitochondrial subfractions. B: Time course of mtNOS activity in IM (□) and SMP (○), and its inhibition by L-NA (■, IM; ●, SMP). Each bar or point represents the mean ± S.E.M. of four to six independent experiments. The units of the two vertical axes in A are identical.
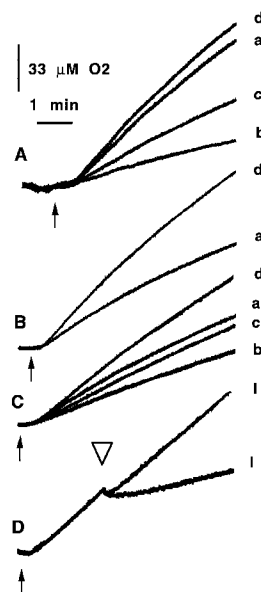
Inhibition by NO of mitochondrial respiration is well established (see [12]). To give further evidence of mtNOS activity and to investigate its putative function(s) we therefore measured mitochondrial respiration under conditions of mtNOS stimulation and inhibition. Fig. 3A shows that respiration of IM is markedly inhibited when mtNOS is stimulated (trace b vs. trace a). This inhibition is partially reversed by 1 mM L-NMMA (trace c). The same result was obtained with BM or MP (data not shown). In the absence of NOS substrates, 1 mM L-NMMA caused a slight increase in the O_2 consumption rate (Fig. 3A, trace d vs. trace a). This stimulation of basal respiration by L-NMMA was more pronounced after five cycles of freeze-thawing of IM (Fig. 3B, trace d vs. trace a). Fig. 3C shows that O_2 consumption by SMP is also inhibitable by mtNOS stimulation (trace a vs. trace b). This inhibition is largely reversed by 1 mM L-NMMA (trace c). Fig. 3C also documents a stimulation by 1 mM L-NMMA of basal SMP respiration (in the absence of NOS substrates) (trace d vs. trace a).

As mentioned, the basal succinate-supported O_2 consumption of BM was suppressed by mtNOS stimulation. Scavenging of NO by 30 μM HbO_2 [19] indeed stimulated this basal O_2 consumption 1.35-fold (not shown). Pretreatment of BM with L-NMMA (1 mM) abolished the stimulation by HbO_2.

Fig. 3D shows that with succinate-supported respiring IM, addition of NOS substrates causes a strong inhibition of O_2 consumption (trace I). When NOS substrates were omitted, one by one, the same inhibition as trace I was achieved (not shown) except when Ca^{2+} was left out (trace II), which led to relief of the inhibition.
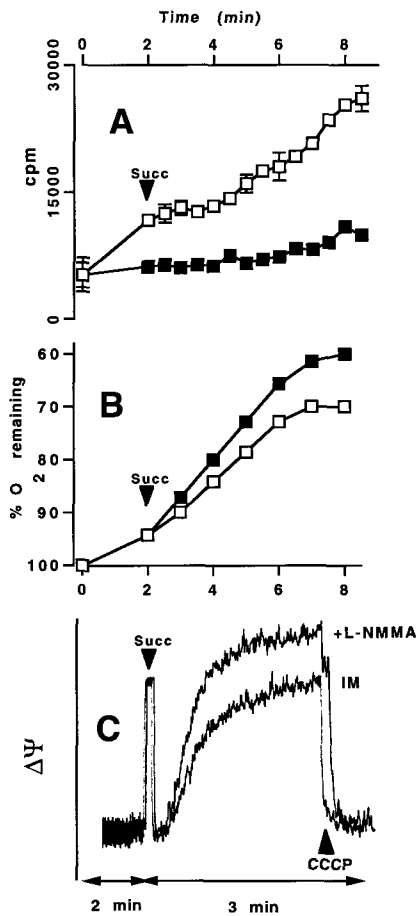
Fig. 4. Basal mtNOS activity, and its effect on mitochondrial respiration and membrane potential. A: Time course of citrulline formation by 1 mg succinate (0.8 mM)-energized IM (□), and its inhibition by 3 mM L-NMMA (■). B: Mitochondrial O_2 consumption measured in parallel with A. Samples of A were take from the incubation mixture used for O_2 consumption measurement. The percentage of oxygen remaining was calculated based on the calibration with a saturated solution of sodium dithionite. C: Effect of 3 mM L-NMMA on ΔΨ formation by IM under the same conditions as in A. At ▼, K$^+$-succinate (Succ), and at ▲ uncoupler (CCCP) were added. Each point represents the mean ± S.E.M. of four independent experiments; the traces are representative of four to six independent experiments.

To investigate further the basal mtNOS activity, IM were incubated for 2 min with L-[^{3}H]arg (30 000–50 000 cpm) and then energized with succinate. Fig. 4A shows L-NMMA (3 mM)-sensitive citrulline formation by these mitochondria. The O_2 consumption measurement (Fig. 4B) performed under the same condition revealed a parallel increase in O_2 consumption upon inhibition of the basal mtNOS activity. The measurement of ΔΨ (Fig. 4C) performed under the same conditions shows that L-NMMA is able to stimulate both the rate and magnitude of ΔΨ formation. L-NMMA added to already K$^+$-succinate (0.8 mM)-energized mitochondria caused an immediate rise in ΔΨ (not shown).

To give further evidence for the Ca^{2+} dependence of mtNOS, we measured citrulline formation in energized IM in the presence of varying amounts of Ca^{2+} (Fig. 5). The activity of the enzyme increases over its basal activity in a Ca^{2+}-dependent manner. A full inhibition by both L-NMMA

(3 mM) and L-NA (5 mM, not shown) was observed at all Ca^{2+} concentrations used.

As shown above, addition of NOS inhibitors increased the O_2 consumption by mitochondria. To rule out a possible direct chemical reaction of the inhibitors with O_2 and to investigate the contribution to mitochondrial O_2 consumption by mtNOS, we blocked succinate-dependent respiration of IM (about 23 nmol of O_2 consumed per min and mg, see Fig. 3) with antimycin A and after 2 min added L-NMMA or L-NA. No alteration in O_2 level was recorded before and after addition of either NOS inhibitor. These findings rule out their direct reaction with O_2, and also show that the contribution of constitutively active mtNOS to the overall mitochondrial O_2 consumption is negligible.

4. Discussion

In the present study, using independent and well-established methods, we show NOS activity in mitochondria. Mitochondrial NOS requires Ca^{2+} and, in a stereo-selective manner, uses L-arginine to produce NO and L-citrulline. The activity is time- and protein concentration-dependent, and is blocked by the L-arginine analogues L-NMMA and L-NA. The spectrophotometric detection of NO requires rupture of the inner mitochondrial membrane, whereas with the citrulline assay NOS activity is detectable with IM, MP, SMP and BM. Several thiol containing compounds, notably glutathione, are present in mitochondria [38]. Thiols can trap NO and thereby conceivably prevent it from reaching the extramitochondrial NO indicator, HbO_2. In experiments not reported here, we found S-nitroso-glutathione, S-nitroso-cysteine and S-nitroso-cysteine-glycine (K. Do et al., unpublished observation) following mtNOS stimulation in IM.

A successful spectrophotometric detection of mtNOS-produced NO required high amounts of SOD. This enzyme lowers the steady-state concentration of superoxide, a radical with which NO eagerly reacts to form peroxynitrite [39–41]. Superoxide is produced by both the mitochondrial respiratory chain [30] and, under certain conditions, e.g. substrate and cofactors limitation, by NOS [40–42]. It is well known that NADPH is present in mitochondria in mM concentrations [42]. The presence in mitochondria of FAD and FMN as components of complexes I and II, respectively, is well established [43].
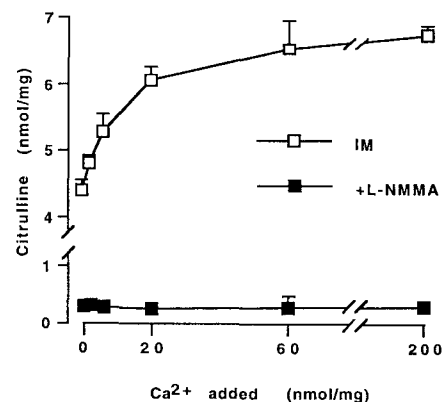

Fig. 5. Calcium dependence of mtNOS activity. Citrulline formation by stimulated mtNOS was measured during 5 min with 1 mg K$^+$-succinate (0.8 mM)-energized IM, in the presence of various Ca^{2+} concentrations, and inhibited by 3 mM L-NMMA. Each point represents the mean ± S.E.M. of four independent experiments.

Although there is some evidence for BH_4 [44] in and arginine uptake by mitochondria [45] the availability of sufficient amounts of these substrates for mtNOS is currently not clear. Xia and Zweier [33] reported a pathway for L-NMMA- and SOD-sensitive superoxide and peroxynitrite generation from iNOS in macrophages depleted of cytosolic L-arginine. Evidence was also presented for nitration of mitochondrial MnSOD by peroxynitrite [46], and we found an increase in nitrotyrosine level in mitochondria following mtNOS activation (M. Shigenaga et al., unpublished observation). Peroxynitrite formation, as a possible consequence of L-arginine and/or BH_4 limitation for mtNOS activity, of superoxide formation by respiratory chain, and of mitochondrial SOD activity, clearly needs to be investigated further.

NOS activity in mitochondria was also shown by measuring citrulline. Treatment of mitochondria with digitonin during MP, and thereby SMP preparations, resulted in increased citrulline formation, compared with IM, presumably because this treatment makes the substrates more easily accessible to mtNOS. Digitonin treatment solubilizes all but the inner mitochondrial membranes. The fact that MP and SMP were separated by centrifugation of washed material rules out a possible contribution of non-mitochondrial NOS to the activity found in MP and SMP. Inhibition by NOS inhibitors of citrulline formation was pronounced and most effective in BM and SMP, followed by MP and IM. This rank order most likely is due to a resistance of the inner mitochondrial membrane to penetration of the inhibitors.

Immunocytochemical studies by others [22,24] have suggested that mtNOS is located at the cristae, the inner, and the outer mitochondrial membrane. Some of the antibodies used in those studies were directed against diaphorases. Since several such enzymes are present in mitochondria [47], there is no consensus that such staining may be valuable for NOS detection in mitochondria [22]. Therefore, the immunochemical characterization of NOS in mitochondria requires some refinement.

We show that mitochondrial O_2 consumption is suppressed, in an L-NMMA-reversible manner, following mtNOS stimulation. This is consistent with the inhibition of mitochondrial respiration by exogenous (non-mitochondrial) NO [10–12,15–20]. Furthermore, we found that L-NMMA stimulates basal mitochondrial respiration, in parallel decreases basal citrulline formation, and increases $\Delta\Psi$. These new findings give further evidence for the presence of a functional NOS in mitochondria and, importantly, also suggest that mtNOS is a regulator of mitochondrial O_2 consumption. The close proximity of mtNOS, reported here, to the complexes of the mitochondrial electron transport chain appears to allow an efficient control of oxidative phosphorylation by mtNOS.

All types of constitutive NOS investigated so far require calmodulin for activity. Whether this is also the case for mtNOS is currently unclear. Addition of calmodulin to BM did not stimulate metHb formation, but mitochondria may have their own calmodulin [48]. In the present study Ca^{2+} was the most effective stimulant for mtNOS activation. In our preparation, isolated rat liver mitochondria contain about 3 nmol Ca^{2+}/mg of protein. Since succinate-energized mitochondria produced NO without additional supplementation, this Ca^{2+} level seems to be sufficient for the basal mtNOS activity. Loading mitochondria with additional Ca^{2+} further stimulated mtNOS activity. Since mitochondria are an impor-

tant cellular Ca^{2+} buffer, and since Ca^{2+} handling by mitochondria is important for the organelles themselves and for cell proliferation, apoptosis and necrosis [49], the possible involvement of mtNOS in these events needs to be investigated.

Stimulation of mtNOS by Ca^{2+} uptake is likely to be part of a feedback loop which prevents overloading of the organelle with Ca^{2+} and allows its release with preserved $\Delta\Psi$. Thus, we have previously shown that peroxynitrite, the stoichiometric product of the reaction of NO and superoxide, stimulates Ca^{2+} release from intact mitochondria [27]. Our present findings suggest that uptake of Ca^{2+} by respiring mitochondria may lead to increased peroxynitrite formation in mitochondria, which in turn causes Ca^{2+} release from intact mitochondria via the pyridine nucleotide-dependent pathway [28] followed by mtNOS deactivation. In contrast to NO, peroxynitrite does not readily inhibit mitochondrial respiration [19]. Mitochondrial superoxide and SOD, by modulating the steady-state concentrations of NO and peroxynitrite, may therefore have a hitherto undetected impact on mitochondrial respiration and respiration-dependent processes, e.g. ATP formation, ion transport and protein import. This, and the possible derangement of mitochondrial respiration in some pathophysiological states such as heart [18] or brain [19] ischemia or neurodegenerative diseases [11], in which the inhibition of mitochondrial respiration plays a central role, will be the subject of further investigations.

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