Production of Nitric Oxide by Mitochondria*

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The production of NO by mitochondria was investigated by electron paramagnetic resonance using the spin-trapping technique, and by the oxidation of oxyhemoglobin. Percoll-purified rat liver mitochondria exhibited a negligible contamination with other subcellular fractions (1–4%) and high degree of functionality (respiratory control ratio = 5–6). Toluene-permeabilized mitochondria, mitochondrial homogenates, and a crude preparation of nitric oxide synthase (NOS) incubated with the spin trap N-methyl-2-gluconic acid–dithiothreitol–FeII produced a signal ascribed to the NO3 spin adduct (g = 2.04; A = 12.5 G). The intensity of the signal increased with time, protein concentration, and l-Arg, and decreased with the addition of the NOS inhibitor Nω-monomethyl-L-arginine. Intact mitochondria, mitochondrial homogenates, and submitochondrial particles produced NO (followed by the oxidation of oxyhemoglobin) at rates of 1.4, 4.9, and 7.1 nmol NO3 per min (mg protein)−1, respectively, with a Km for l-Arg of 5–7 μM. Comparison of the rates of NO3 production obtained with homogenates and submitochondrial particles indicated that most of the enzymatic activity was localized in the mitochondrial inner membrane. This study demonstrates that mitochondria are a source of NO3, the production of which may effect energy metabolism, O2 consumption, and O2 free radical formation.

Nitric oxide (NO)3 is a free radical generated in biological systems by nitric oxide synthases (NOS). Because of its effect on neurotransmission, vasodilation, and immune response (1–3), NO plays an important role in physiology, pathology, and pharmacology.

Studies with brain tissue and macrophage lysates have shown that NOS is localized exclusively in the soluble fraction (3–6), and recent studies have indicated that the majority (>80%) of bovine endothelial NOS activity is bound to the particulate fraction of cell homogenates (7, 8). Because the particulate fraction used in the studies was expected to contain plasma membranes, as well as microsomes, and, possibly, intracellular organelles, the actual subcellular location of the activity remained to be determined. Other lines of evidence have indicated the presence of NOS in the perinuclear region, in discrete regions of the plasma membrane of cultured endothelial cells, and in intact blood vessels (9, 10); immunocytochemical studies have revealed the presence of a NOS, or an antigenically related protein, in mitochondria isolated from different tissues (11–13). The predominant association of this mtNOS with the mitochondrial membrane (11, 12), and its co-localization with succinate dehydrogenase, a mitochondrial marker of the inner membrane (13), suggested that this enzyme has a particulate localization.

These studies as well as the presence of substrates and cofactors in mitochondria required for NOS activity such as l-arginine (l-Arg), l-Arg transporters, Ca2+, calmodulin, NADPH, and the availability of O2, led us to postulate mitochondria as a potential source of NO3 production.

Following the use of a specific spin-trapping agent and the controlled oxidation of oxyhemoglobin, NO3 production was detected in purified mitochondrial preparations (intact mitochondria, permeabilized mitochondria, mitochondrial homogenates, and submitochondrial particles) and crude preparations of NOS (crude fraction) obtained from rat liver.

Given the important implication of a mitochondrial production of NO3 for energy conservation mechanisms and free radical production, such production may serve as the basis for a new understanding of biochemical regulation, based on the ubiquitous distribution of mitochondria and the diffusibility of NO3 through cellular membranes.

MATERIALS AND METHODS

EDTA, EGTA, sodium succinate, sodium malate, sodium glutamate, mannitol, ADP, succrose, HEPES, bovine serum albumin (fatty-acid free), and CHAPS were purchased from Sigma. Catalase, horseradish peroxidase (grade I), and superoxide dismutase were obtained from Boehringer Mannheim. The spin trap, N-methyl-2-glucamine–dithiocarbamate–FeII (MGD), was purchased from the Oklahoma Medical Research Foundation (Oklahoma City, OK). 4-Hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO or TEMPOL) was obtained from Aldrich. Oxymyoglobin was prepared from 1 mM of commercially available horse heart myoglobin in 0.1 M Hepes buffer, pH 7.4 (degassed with helium), mixed with a slight molar excess of sodium dithionite. The sample was aerated, and excess dithionite was removed by gel filtration using a Sephadex G-25 column (14).

Biological Materials

Liver mitochondria were isolated from adult Wistar rats (180–200 g) by differential centrifugation, essentially as described in Ref. 15. The livers from rats (anesthetized with pentobarbital or decapitated) were excised, washed with 0.25 M sucrose, and homogenized (1/10, w/v) using MSHE (0.22 M mannitol, 0.07 M sucrose, 0.5 mM EGTA, 0.1% bovine...
Cytochrome c oxidase activity was expressed as nanomoles of cytochrome c oxidized/min/mg of protein; glucose-6-phosphatase, 5'-nucleotidase, and acid phosphatase activities were expressed as nanomoles of inorganic phosphate/min/mg of protein; catalase activity was expressed as micromoles of H$_2$O$_2$ consumed/min/p mg of protein (see Ref. 26 and references therein). Recovery is the ratio of specific activities of an enzyme in mitochondria and in the homogenate (Ratio) corrected by the protein recovered in the mitochondrial fraction (Recovery%).

| Enzyme                      | Location      | Specific activity | Ratio     | Recovery |
|-----------------------------|---------------|-------------------|-----------|----------|
|                             | Homogenate    | Mitochondria      |           |          |
| Cytochrome oxidase          | Mitochondria  | 82.5              | 495       | 6        | 18       |
| Glucose-6-phosphatase       | Microsomes    | 35                | 7         | 0.2      | 0.6      |
| 5'-Nucleotidase             | Plasma membrane| 42               | 17        | 0.4      | 1.2      |
| Acid phosphatase            | Lysosomes     | 77                | 100       | 1.3      | 3.9      |
| Catalase                    | Peroxisomes   | 350               | 2         | 0.6      | 1.8      |

Production by Mitochondria

Table I

Enzymatic characterization of isolated rat liver mitochondria

Cytochrome c oxidase activity was expressed as nanomoles of cytochrome c oxidized/min/mg of protein; glucose-6-phosphatase, 5'-nucleotidase, and acid phosphatase activities were expressed as nanomoles of inorganic phosphate/min/mg of protein; catalase activity was expressed as micromoles of H$_2$O$_2$ consumed/min/p mg of protein (see Ref. 26 and references therein). Recovery is the ratio of specific activities of an enzyme in mitochondria and in the homogenate (Ratio) corrected by the protein recovered in the mitochondrial fraction (Recovery%).

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| Glucose-6-phosphatase | Microsomes   | 35                | 7     | 0.2      | 0.6      |
| 5'-Nucleotidase  | Plasma membrane| 42                | 17    | 0.4      | 1.2      |
| Acid phosphatase | Lysosomes      | 77                | 100   | 1.3      | 3.9      |
| Catalase        | Peroxisomes    | 350               | 2     | 0.6      | 1.8      |

medium used with permeabilized mitochondria consisted of 225 mM sucrose, 5 mM MgCl$_2$, 20 mM KCl, 10 mM potassium phosphate, 0.1 mM NADPH, 1 mM CaCl$_2$, 10 μM reduced tetrahydrobipterin, 20 mM Hepes/KOH, pH 7.4, plus 8.5% (v/v) polyethylene glycol 8000.

Protein Determination

Protein was determined by the Lowry assay (24) using bovine serum albumin as standard.

Data Evaluation

All assays were done in duplicate and were repeated five to eight times in separate experiments using 2–4 rats/experiment. Data are presented as mean ± S.E., in which the S.E. were between 10 and 12% of the mean values.

RESULTS

Assessment of Purity and Functionality of Rat Liver Mitochondria—Rat liver mitochondria were isolated by differential centrifugation (15), purified by Percoll centrifugation (16), and washed with high ionic strength solutions. This procedure allowed the efficient removal of contaminating organelles, broken mitochondria, arginase (25), and adsorption artifacts, yielding a highly purified preparation. This is supported by the low degree of non-mitochondrial contamination (1–4%; Table I), which was comparable with, and in some cases less than, that obtained with other purification procedures (18, 26, 27).

Mitochondria isolated using this procedure differ from those obtained by differential centrifugation in that the former exhibited a higher respiratory control ratio, indicating functional integrity and membrane intactness (Table II).

Detection of Nitric Oxide in Mitochondrial Preparations by Spectroscopic Techniques: Detection of NO by Spin Trapping/EPR—Toluene-treated mitochondria with an increased permeability for the spin trap and external NADPH were incubated with the spin trap (MGD)/Fe$^{II}$ for 1 h at room temperature. A weak EPR signal consisting of a triplet ($\alpha_N$ = 12.5 G; $\beta_{iso}$ = 2.04; intensity ratio 1:1:1; Fig. 1A) was assigned to the (MGD)/Fe$^{II}$-NO$^-$ complex (21, 22) by comparison with the signal obtained with the NO$^-$ donor, nitrosoglutathione (Fig. 1E).

In addition to the triplet, a line from a quartet signal was present assigned to the (MGD)/Cu$^{II}$-NO$^-$ complex produced by the reaction of free Cu$^{II}$, in the homogenate or in the reaction solution, with the excess of MGD. The addition of L-Arg increased the signal by 30% (Fig. 1B), whereas N$^O$-monomethylarginine (NMMA), the competitive inhibitor of NOS (30), decreased the signal by 50% (Fig. 1C) and 20% (Fig. 1D) in the absence and presence of L-Arg, respectively. Based on its sensitivity to NMMA, the formation of the (MGD)/Fe$^{II}$-NO$^-$ signal in the biological sample without L-Arg suggests an endogenous pool of Arg capable of sustaining NO$^-$ production through an NOS-catalyzed reaction (Fig. 1C).

The NMMA-insensitive EPR signal suggested the presence of a labile pool of NO$^-$ (2). Of note, the addition of 5

C. Giulivi, unpublished observations.
mu g/ml calmodulin and/or 1 mM Ca2+ did not significantly affect the signal intensity, indicating that NOS was fully active with the cofactors present in our preparations.

Toluene-treated rat liver mitochondria, incubated with the spin trap for 8 h to increase the substrate and inhibitor concentrations in the mitochondrial matrix, exhibited signal intensities 2.3- to 2.7-fold higher than those found at 1 h, with and without L-Arg addition, respectively (Fig. 2, A and B). Preincubation of mitochondria with NMMA inhibited the signal formation by 50% and 15% in the absence and presence of L-Arg, respectively (Fig. 2, C and D). A weak (MGD)2/FeII-NO signal was also noted in the absence of mitochondrial homogenate (data not shown); this signal is likely to originate from NO\textsuperscript{+} diffusion from ambient air (about 0.1 ppm).

Mitochondrial homogenates or a crude fraction of NOS incubated for 1 h with L-Arg and the spin trap showed the same three-line EPR spectrum observed with toluene-permeabilized mitochondria (Table III). The EPR signal intensities obtained with crude fraction were 5- to 7-fold higher than those observed with permeabilized mitochondria. The signal intensities were increased by L-Arg supplementation and decreased by NMMA addition to different extents, depending on the specific biological preparation (Table III). Similar results were obtained using L-N\textsuperscript{5}-(1-iminoethyl)ornithine (NIO), another NOS inhibitor (data not shown). The high concentrations of NMMA required in these experiments are indicative of the competitive kinetics of the inhibition of NOS by NMMA in the presence of an endogenous pool of L-Arg, the latter most likely sustained by proteolytic activities present in the samples.

The iron-nitrosyl complex signal intensities obtained with toluene-treated rat liver mitochondria after 1 h of incubation showed a linear dependence on protein concentration. Double integration of the EPR signals and interpolation of calibration curves of the EPR signals allowed the quantification of the EPR signals. This quantification permitted the calculation of an NO\textsuperscript{+} production rate of 48 ± 2 nmol/mg mitochondrial protein (r = 0.99). The NO\textsuperscript{+} experimentally detected by EPR accounted for approximately 16% of the rate of metmyoglobin formation, even when an excess of spin trap was used. This underestimation may be due to the metabolism of the spin trap by mitochondrial prep-

### Table II

| Isolation procedure | Percoll centrifugation | Differential centrifugation |
|---------------------|------------------------|-----------------------------|
| State 3             | (ng-atoms O/min/mg protein) | 133 ± 10          |
| State 4             | (ng-atoms O/min/mg protein) | 21 ± 2            |
| RCR                 | Malate-glutamate       | 4.5 ± 0.5         |
|                     | Succinate              | 5.9 ± 0.5         |
| P/O                 | Malate-glutamate       | 2.8 ± 0.1         |
|                     | Succinate              | 2.8 ± 0.2         |

**FIG. 1.** EPR spectra of permeabilized mitochondria supplemented with NOS substrates and inhibitors after 1 h of incubation. The reaction medium contained 225 mM sucrose, 5 mM MgCl\textsubscript{2}, 20 mM KCl, 10 mM potassium phosphate, 0.1 mM NADPH, 1 mM CaCl\textsubscript{2}, 10 \mu M reduced tetrahydrobioperin, 20 mM Hepes/KOH, pH 7.4 plus 8.5% (v/v) polyethylene glycol 8000, 30 mM (MGD)\textsubscript{2}/Fe\textsuperscript{II}, and (5.2 mg of protein) toluene-treated mitochondria. These samples were incubated for 1 h at room temperature without (A and C) and with (B and D) 1 mM L-Arg, plus 10 mM NMMA (C and D). The EPR spectra of the iron-nitrosyl complex (●) or the (MGD)\textsubscript{2}/Cu\textsuperscript{II} complex (○) were recorded at room temperature operating at 9.77 GHz in an EPR spectrometer (Bruker ECEX 06). Instrument settings: modulation frequency, 100 kHz; modulation amplitude, 2.9 G; sweep scan 0.9 G/s; sweep width, 150 G; microwave power, 0.10 milliwatts; time constant, 0.6 s; receiver gain, 2.5 × 10\textsuperscript{5}. EPR spectrum of the (MGD)\textsubscript{2}/Fe\textsuperscript{II}-NO\textsuperscript{+} complex (E) originated from a reaction mixture containing 0.1 mM nitrosothioglutathione, 30 mM (MGD)\textsubscript{2}/Fe\textsuperscript{II}, 0.5 mM Hepes buffer, pH 7.4. Nitrosothioglutathione was synthesized in situ by the addition of 0.2 mM sodium nitrite in 0.1 M HCl and 0.2 mM reduced glutathione in 1 mM HEPES buffer, pH 7.4. Spectrum E was recorded with a receiver gain of 3.2 × 10\textsuperscript{5} and a time constant of 0.2 s. All the EPR spectra were recorded in bottom-sealed Pasteur pipettes.

**FIG. 2.** EPR spectra of permeabilized mitochondria supplemented with NOS substrates and inhibitors after 8 h of incubation. Toluene-permeabilized mitochondria (5.2 mg of protein) were added to reaction mixtures without (A and C) and with (B and D) 1 mM L-Arg, plus 10 mM NMMA (C and D) and then incubated for 8 h at room temperature. The EPR conditions and reaction mixtures were described under Fig. 1.

### Table III

**EPR signal intensities of the (MGD)\textsubscript{2}/Fe\textsuperscript{II}-NO\textsuperscript{+} for different mitochondrial preparations**

| Additions | Toluene-treated mitochondria | Homogenate (1 h) | Crude fraction (1 h) |
|-----------|-------------------------------|------------------|----------------------|
| None      | 1.0                           | 2.3              | 0.7                  | 4.98                 |
| L-Arg     | 1.30                          | 3.5              | 1.00                 | 9.75                 |
| NMMA      | 0.5                           | 1.2              | 0.4                  | 2.0                  |
| L-Arg + NMMA | 1.05                         | 3.0              | 0.7 (0.5)            | 4.9 (2.34)          |
ations to EPR silent species, probably of the type (MGD)Fe\textsuperscript{II}-NO\textsuperscript{X}, where X = halogen ions or NO\textsubscript{2}, as has been described for diethylthiocarbamate (31, 32). This notion is strengthened by the similar recovery (12%) of an EPR signal of a synthetic iron-nitrosyl complex (formed by incubating sodium nitroprusside, an NO\textsuperscript{•} donor, and the spin trap) and the decrease in the signal intensities with protein concentrations above 12 mg.

Measurement of NO\textsuperscript{•} Production by the Oxidation of Oxymyoglobin—The production of NO\textsuperscript{•} in the presence of l-Arg was measured in intact mitochondria, mitochondrial homogenates, and submitochondrial particles by following the NMMA-sensitive oxidation of oxymyoglobin to metmyoglobin (Fig. 3). The NMMA-insensitive rates of oxymyoglobin oxidation were 20–20% of the total rate of metmyoglobin formation using mitochondria and mitochondrial homogenates, and 30% when using SMP. The higher unspecified oxidation in the latter instances may be attributed to a direct oxidation of oxymyoglobin by a component of the respiratory chain.\textsuperscript{3}

The rates of NO\textsuperscript{•} production increased linearly with the protein concentration of mitochondrial preparations (Fig. 3). The specific rates were 1.36, 4.9, and 7.1 nmol × (min·mg protein)\textsuperscript{-1} for intact mitochondria, mitochondrial homogenates, and submitochondrial particles, respectively. The activities obtained with mitochondrial homogenates and submitochondrial particles were higher than those obtained with intact mitochondria because the former were assessed under conditions for optimal NOS activity. A comparison of the rates of SMP and mitochondrial homogenates under identical conditions indicated that most of the activity was detected in the mitochondrial inner membrane (considering that 30% of the rat liver mitochondria protein corresponds to the inner membrane fraction (33), 25–35% of the particles had the “right side-in” conformation (34), and 80% of the activity (experimentally determined) was recovered after the sonication procedure) suggesting that NOS could be mainly (60–80%) localized in this membrane fraction.

The production of NO\textsuperscript{•} by intact mitochondria was followed by the NMMA-sensitive oxidation of oxymyoglobin in the presence of l-Arg (Fig. 4A). The rapid onset of the production was indicative of a fast transport of L-Arg into mitochondria, consistent with the reported L-Arg carriers found in isolated mitochondria and mitochondrial homogenates, and 30% when using SMP. The higher unspecified oxidation in the latter instances may be attributed to a direct oxidation of oxymyoglobin by a component of the respiratory chain.\textsuperscript{3}

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A slow rate of NO\textsuperscript{•} production by intact mitochondria was also detected in the absence of added l-Arg (11 nmol/min; Fig. 4A). This rate may reflect a slow but constant production of NO\textsuperscript{•} by mitochondria using endogenous l-Arg, which may be increased under special pathophysiological conditions, such as

\[ \text{FIG. 3. NO}^\cdot \text{ production by mitochondrial homogenates, submitochondrial particles, and intact mitochondria. NO}^\cdot \text{production by intact, purified mitochondria (●) was measured in a reaction mixture containing 0.225 M sucrose, 5 mM MgCl}_2, 20 mM KCl, 10 mM potassium phosphate, 10 mM succinate, 20 mM Hepes/KOH, pH 7.4 supplemented with 50 \mu M oxymyoglobin. Mitochondrial homogenates (○) or SMP (▲) were incubated in 1 mM l-Arg, 1 mM MgCl}_2, H}_2O, 1 mM CaCl}_2, 0.1 mM NADPH, 12 \mu M tetrahydrobiopterin, in 0.1 M Hepes buffer, pH 7.5, supplemented with 50 \mu M oxymyoglobin. The transition oxy [→] metmyoglobin was followed at 581–592 nm during the first 3 min. Parallel experiments were performed in samples preincubated with 10 mM NMMA.} \]

\[ \text{FIG. 4. NO}^\cdot \text{ production by intact, coupled mitochondria and toluene-permeabilized mitochondria. Intact, coupled mitochondria (panel A) or toluene-permeabilized mitochondria (panel B) at 1 mg of protein concentration were incubated in the respective reaction mixtures (0.225 M sucrose, 5 mM MgCl}_2, 20 mM KCl, 10 mM potassium phosphate, 10 mM succinate, 20 mM Hepes/KOH, pH 7.4, for mitochondria, and 225 mM sucrose, 5 mM MgCl}_2, 20 mM KCl, 10 mM potassium phosphate, 0.1 mM NADPH, 1 mM CaCl}_2, 10 \mu M reduced tetrahydrobiopterin, 20 mM Hepes/KOH, pH 7.4, plus 8.5% (w/v) polyethylene glycol 8000 for permeabilized mitochondria). All incubations contained 50 \mu M oxymyoglobin (see "Materials and Methods"). Inset, double-reciprocal plot of the rates of NO}^\cdot \text{ production and l-Arg concentration. Data were obtained from the respective main figures.} \]

\[ ^3 \text{C. Giulivi and E. Cadenas, unpublished observations.} \]
Inhibition of NO production by intact mitochondria

Intact rat liver mitochondria (0.52 mg of protein/ml) were incubated in 1 ml of the reaction mixture containing 30 μM L-arginine and 50 μM oxyhemoglobin. The suspensions were supplemented with 0.1 mM inhibitors, and the rate of NO production was quantified by measuring the oxidation of oxyhemoglobin at 581–592 nm after 5 min of incubation. Mitochondria were preincubated with the inhibitors for 10 min, and then L-Arg was added.

| Additions | Inhibition of NO production |
|-----------|-----------------------------|
|           | No incubation | Preincubation |
| D-Arginine| 1.2           | 2.5           |
| NIO       | 6.2           | 43.2          |
| NMMA      | 100.0         | 100.0         |

Production of NO by submitochondrial particles

SMP (0.4 mg of protein) were incubated in 0.1 M HEPES buffer, pH 7.4, for 15 min with 5 mM NMMA, and the reaction started by L-Arg addition. The rate of NO production was measured in the presence of 50 μM oxyhemoglobin, and the formation of metmyoglobin was followed spectrophotometrically at 581–592 nm. Specific activities were calculated as total activity minus the activity in the presence of NMMA. Other conditions are explained under “Materials and Methods.”

| Additions | Total activity | Specific activity |
|-----------|---------------|------------------|
|           | nmol/min      | nmol NO/min/mg protein |
| NMMA (5 mM) | 1.19        | 0.0              |
| Plus L-Arg (1 mM) | 1.36        | 0.4              |
| Plus L-Arg (10 mM) | 4.00        | 7.0              |

DISCUSSION

The following lines of evidence offer support for the mitochondrial generation of NO.

First, the negligible contamination of the mitochondrial preparations with other subcellular fractions (Table I) and the integrity and functionality of these preparations (Table II) support the production of NO by mitochondria.

Second, the production of NO by mitochondria was demonstrated by two different spectroscopic assays: the formation of metmyoglobin, and spin trapping/EPR. To rule out species other than NO reacting with oxyhemoglobin (41–45), the rate of oxidation of oxyhemoglobin was monitored under controlled conditions (with catalase and superoxide dismutase and measuring sensitivity to NMMA). However, because some NO-derived oxides may still be able to produce this reaction, unequivocal identification of NO was furnished by using the spin trap MGD, the iron-nitrosyl complex ESR signal of which is considered a “fingerprint” of NO (21, 22). The advantages of this technique (selectivity of the spin trap toward NO and the lack of toxicity of the spin trap in biological systems) were initially limited by the free access of the spin trap to the biological source, the low recovery of the iron-nitrosyl adduct, and the presence of quenchers of NO (e.g. hemoproteins, [Fe-S] clusters) that effectively compete with the spin trap MGD. These limitations were overcome by using millimolar concentrations of the spin trap (to effectively compete with other possible NO quenchers) with tolune-treated mitochondria and mitochondrial homogenates (to allow free access of the spin trap to the biological source of NO). The use of submitochondrial particles and mitochondrial homogenates, albeit less physiologically relevant than intact mitochondria, devoid of the limitations noted above permitted the selection of optimal conditions for NO production by mitochondria.

Third, the production of NO is catalyzed by an enzyme, likely a NOS isoform, located at the mitochondrial inner membrane. This was inferred from three separate lines of evidence. (i) NO production was modulated by NOS substrates (L-Arg) and inhibitors (NMMA, NIO, and D-Arg); (ii) the rate of NO production by mitochondria and SMP versus L-Arg concentration followed a similar pattern to that described for NOS purified from different tissues; (iii) the higher specific activities in SMP or crude fraction (about 2 and 10 times higher, respectively) than those obtained with mitochondrial homogenates or permeabilized-mitochondria were indicative of an enzymatic activity located at the inner membrane. Conclusive evidence that a NOS isoform was responsible for the NO production was provided by the purification and characterization of the enzyme from purified rat liver mitochondria reported in the accompanying paper (46).

The rate of NO production by rat liver mitochondria reported herein and by others is similar to that of O2 (about 1.2 nmol of O2/min/mg of protein, equivalent to 0.6 nmol of H2O2/min/mg of protein; Ref. 33). At saturating concentrations of L-Arg, a steady-state concentration of NO in the range of 0.1–0.5 μM may be sustained. These values contemplate the reaction of NO with cytochrome oxidase or with O2 as the main catabolic pathways. Of note, this level of NO is biologically relevant because it is in the range of those concentrations reported to inhibit respiration in synaptosomes (47) and intact mitochondria (48). Given the role of NO as a cellular messenger, transmitter, and regulator (1–3), it could be hypothesized that this inhibition (or modulation) of mitochondrial respiration by NO may represent a novel biochemical pathway regulating the supply of O2 and energy to tissues under dynamic conditions.

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