The Modulation of Oxygen Radical Production by Nitric Oxide in Mitochondria*

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Biological systems that produce or are exposed to nitric oxide (NO) exhibit changes in the rate of oxygen free radical production. Considering that mitochondria are the main intracellular source of oxygen radicals, and based on the recently documented production of NO by intact mitochondria, we investigated whether NO, produced by the mitochondrial nitric-oxide synthase, could affect the generation of oxygen radicals. Toward this end, changes in H₂O₂ production by rat liver mitochondria were monitored at different rates of endogenous NO⁻ production. The observed changes in H₂O₂ production indicated that NO⁻ affected the rate of oxygen radical production by modulating the rate of O₂ consumption at the cytochrome oxidase level. This mechanism was supported by these three experimental proofs: 1) the reciprocal correlation between H₂O₂ production and respiratory rates under different conditions of NO⁻ production; 2) the pattern of oxidized/reduced carriers in the presence of NO⁻, which pointed to cytochrome oxidase as the crossover point; and 3) the reversibility of these effects, evidenced in the presence of oxymyoglobin, which excluded a significant role for other NO⁻-derived species such as peroxynitrite. Other sources of H₂O₂ investigated, such as the aerobic formation of nitrosoglutathione and the GSH-mediated decay of nitrosoglutathione, were found quantitatively negligible compared with the total rate of H₂O₂ production.

Biological systems of diverse complexity, when exposed to NO⁻ or stimulated to produce NO⁺, usually present changes in the rate of oxygen free radical production (Ref. 1 and references therein). Decreases in the rate of oxygen free radical production are often attributed to the diffusion-controlled reaction between O₂⁻ and NO⁺, which yields peroxynitrite. Increases in reactive oxygen species (ROS) production are usually associated with the damage and/or inactivation of mitochondrial components by peroxynitrite or peroxynitrite-like species.

Considering that under physiological conditions mitochondria constitute the main intracellular source of oxygen free radicals (2, 3) and that these organelles can produce NO⁺ (4–8), it becomes of interest to examine whether the production of mitochondrial ROS is affected by endogenous NO⁺. The accurate documentation of the rates of production of nitrogen and oxygen radicals is an essential step to understanding the role of these interactions in different, more complex pathophysiological situations and underlines the relevance of studying the mechanisms behind these processes in relatively simpler models. Furthermore, transient changes in ROS production have become an area of intense research given the growing interest in the role of ROS as mediators of signal transduction pathways, organ preconditioning (9), and apoptotic processes (10).

In this study, we examined the rates of O₂ free radical production by intact mitochondria under various conditions of endogenous NO⁻ production; different mechanisms underlying the NO⁻-O₂ interactions were quantitatively investigated. Herein, we demonstrated that mitochondria could generate O₂⁻ and NO⁺; however, unlike other biological systems, no irreversible damage to mitochondrial components was observed. The enhanced ROS production detected under NO⁻-producing conditions was attributed to an increase in the steady-state level of reduced components of the respiratory chain, whereas a small contribution was attributed to the aerobic decay of S-nitrosoglutathione (GSNO) in the presence of GSH. Finally, the implications of these results were discussed in terms of the ability of NO⁻ to modulate the rates of oxygen free radical production and/or consumption and the pathophysiological consequences associated with this process.

EXPERIMENTAL PROCEDURES

Chemicals and Biochemicals

EDTA, EGTA, sodium succinate, sodium malate, sodium glutamate, mannitol, sucrose, HEPES, bovine serum albumin (fatty acid free), L-arginine, antimycin, 2× crystallized scopoletin, and desferrioxamine mesylate were purchased from Sigma. Catalase and horseradish peroxidase (Grades I) were obtained from Roche Molecular Biochemicals. Oxymyoglobin was obtained as described before (5, 6). All other reagents were of analytical grade.

Biological Materials

Liver mitochondria were isolated from adult Wistar rats by differential centrifugation and purified through Percoll centrifugation (5). This procedure yielded intact mitochondria minimally contaminated with other subcellular compartments (5). Essentially, male rats (body weight of 180–220 g) were anesthetized using a CO₂ chamber. The livers were quickly removed; washed with 0.22 m mannitol, 70 mM sucrose, 0.5 mM EGTA, 2 mM Heps, and 0.1% defatted bovine serum albumin, pH 7.4 (Buffer A); and homogenized in a 10:1 buffer to liver w/w ratio. Large cell debris and nuclei were pelleted by centrifuging at 600 × g for 5 min in a Sorvall SS34 rotor. This supernatant was filtered through two layers of cheesecloth to remove fat. Mitochondria were pelleted by centrifuging the supernatant for 10 min at 10,300 × g in the same rotor. After suspending the pellet in 5 ml of the previous buffer, the mitochondria were washed three times and finally resuspended in 2 ml of 50 mM potassium phosphate (pH 7.0).
Oxygen and Nitrogen Radicals in Mitochondria

Biochemical Analyses

Oxygen Consumption—The oxygen uptake of mitochondria was measured using a Clark-type O2 electrode from Hansatech (King’s Lynn, UK) at 30 °C (5, 7). Intact, purified mitochondria (0.5–2 mg of protein/ml) were maintained in 1 ml of reaction medium in the presence of 10 mM sucinate as substrate and, where indicated, different amounts of l-Arg or Nω-monomethyl-l-arginine (NMMA). NOz Detection—The production of NOz by mitochondria (1–2 mg/ml) was evaluated as the change in the absorbance at 581–592 nm at 22 °C using a dual wavelength, double beam SLM-Amino DW-2C UV-visible spectrophotometer. The sample cuvette contained 2 ml of reaction medium with 13 mM L-arginine, 10 mM sucinate, and 0.1 mM l-arginine under constant stirring. When NMMA was used in the incubation, the protein concentration was 3–5 mg/ml. Ten μM catalase and superoxide dismutase were also present in the reaction medium to avoid unspecific side reactions (namely, the reaction of NOz with peroxynitrite/hydrogen peroxide); however, no significant changes were found with or without these enzymes. The rates of NOz production were taken during the first 4–5 min when the ratios of myoglobin:catalase (in heme):NOz were 6.5 ± 0.2 and 2.8 ± 0.1, respectively, assessed in 0.225 mM sucrose, 5 mM MgCl2, 20 mM KCl, 10 mM potassium phosphate, and 20 mM Hepes/KOH, pH 7.4 (reaction medium) supplemented with 10 mM succinate and 0.25 mM ADP.

FIG. 1. Nitric oxide production by l-arginine-supplemented mitochondria. The rate of NOz production was followed by the oxidation of oxymyoglobin or by the production of L-citrulline (not shown), as described under “Experimental Procedures.” A, the velocity of NOz production versus l-Arg concentration with no (closed circles), 30 μM (closed triangles), and 75 μM (open circles) NMMA. B, from the slopes of reciprocal plots of velocity versus [l-Arg], the apparent Km was calculated at each concentration of NMMA. These values were plotted versus the [NMMA].

RESULTS AND DISCUSSION

Endogenous Nitric Oxide Modulates Hydrogen Peroxide Production by Mitochondria—The addition of increasing concentrations of l-Arg to intact, coupled mitochondria in State 4 (using either glutamate-malate or succinate) stimulated the production of NOz (Fig. 1A). The initial velocity plotted versus l-Arg concentration represented a right rectangular hyperbola; the Vmax and Km values were 1.5 nmol of NOz × (min · mg protein)−1 and 5 μM, respectively. Comparable results were obtained by measuring L-citrulline, cysynthesized with NOz by NOS (Vmax = 1.2 ± 0.2 nmol of L-citrulline/min × mg of protein and Km = 8 μM). The rate of NOz production was negligible at l-Arg concentrations of 50–100 × Km. This effect was accompanied by an increase in the rate of oxygen consumption (3–5 times), found to be resistant to ADP stimulation, and comparable with the increase obtained in the presence of carbonyl cyanide p-trifluoromethoxyphenylhydrazone. These experimental observations indicated that the lack of NOz production at high concentrations of l-Arg was attributed to the uncoupling of mitochondria by this amino acid.

The rates of NOz production in mitochondria, maintained in State 4, were decreased significantly when NMMA was included in the reaction mixture (Fig. 1A). The addition of this compound did not affect the Vmax but increased the apparent Km values (15 and 30 μM, respectively). These results indicated that this compound is acting as a competitive inhibitor of mitochondrial NOS, as has been shown by other reports (17–19). The Km was calculated from the slope of a plot of Km(app) versus [NMMA] (Fig. 1B), resulting in a value (15 μM) similar to that reported for mouse-inducible NOS (13 μM) (18). Even at high concentrations of NMMA (0.5 mM), a slow rate of NOz was detectable (0.02 nmol of NOz × (min · mg protein)−1) not accompanied by L-citrulline production. This slow rate has been attributed to the nonenzymatic release of NOz from GSNO in the presence of reduced glutathione and superoxide dismutase.2

To examine whether endogenous NOz affected the mitochondrial production of oxygen free radicals, the production of H2O2 was...
and the oxygen consumption of intact mitochondria were assessed under different conditions of NO\(^+\) generation. Mitochondria were supplemented with either malate-glutamate or succinate during the experiments to ensure a nonlimiting pool of NADPH, required for the activity of NOS.

The addition of L-Arg to intact, coupled substrate-supplemented mitochondria increased the rate of H\(_2\)O\(_2\) production in a dose-response manner (Fig. 2A). The concentrations of L-Arg required for the half-maximal effect were 29.6 \(\mu\)M (glutamate-malate) and 34 \(\mu\)M (succinate). At saturating concentrations of L-Arg, the rate of H\(_2\)O\(_2\) production increased by 42\% (glutamate-malate) and 58\% (succinate), whereas the respiratory rates decreased 33\% (glutamate-malate) and 27\% (succinate) (Fig. 2B).

The concentrations of L-Arg required for the 50\% inhibition of the respiratory rates were 24.7 \(\mu\)M (glutamate-malate) and 31.5 \(\mu\)M (succinate). The effects observed on the respiratory rates and H\(_2\)O\(_2\) production were completely reversed by adding 0.2–1 mM oxyhemoglobin to the reaction mixture (not shown). These latter experiments might wrongly indicate that the rate of H\(_2\)O\(_2\) production was decreased by the reaction of H\(_2\)O\(_2\) with oxyhemoglobin via its pseudocatalatic/peroxidatic activity, without the participation of NO\(^+\). This possibility seemed unlikely based on the following evidence. First, the rate constants for the reaction of oxyhemoglobin or oxymyoglobin with H\(_2\)O\(_2\) in their peroxidatic and catalatic activities are 4–5 orders of magnitude smaller than those corresponding to the reaction of oxyhemoglobin with NO\(^+\) (21–23), indicating that this molecule will preferentially react with oxyhemoglobin rather than with H\(_2\)O\(_2\). Second, the addition of NMMA, which blocks the production of NO\(^+\) by mitochondrial NOS (independently of the presence of oxyhemoglobin) decreased the rate of H\(_2\)O\(_2\) production, excluding the role of oxyhemoglobin in the modulation of H\(_2\)O\(_2\) production by NO\(-\)producing mitochondria (see below).

The addition of NMMA to L-Arg- and succinate-supplemented mitochondria decreased the rate of H\(_2\)O\(_2\) production and increased the respiratory rates in a dose-response manner (Fig. 3, A and B, respectively). The concentrations of NMMA required for the half-maximal rate of H\(_2\)O\(_2\) production were 13.9 (glutamate-malate) and 13.8 \(\mu\)M (succinate); at saturating concentrations of NMMA, the rate of H\(_2\)O\(_2\) production was decreased 42\% (malate-glutamate) and 37\% (succinate). The concentrations of NMMA required for the half-maximal respiratory rates were 15.7 (glutamate-malate) and 10.2 \(\mu\)M (succinate); at saturating concentrations of NMMA, the respiratory rates were increased by 85\% (malate-glutamate) and 39\% (succinate). The effects observed on the respiratory rates and H\(_2\)O\(_2\) production mediated by NMMA were completely reversed by adding increasing amounts of L-Arg (not shown).

The reciprocal association between ROS and NO\(^+\) production with the O\(_2\) uptake (Figs. 1–3, Table I) and the similar concentrations of NMMA and L-Arg required for the half-maximal effects for the NO\(^+\) production, respiratory rates, and H\(_2\)O\(_2\) production (Figs. 1–3) indicated that these events were biochemically linked, acting at a common site. The involvement of NO\(^+\) in these effects was supported by the reversibility of the effects observed with NMMA (upon the addition of L-Arg) and L-Arg (upon the addition of oxymyoglobin). These results also precluded a significant role for peroxynitrite because this species causes irreversible damage of respiratory chain components (24). Furthermore, the fact that oxymyoglobin reversed the modulation of the O\(_2\) uptake and ROS production not only excluded ONOO\(^-\) but also the involvement of other NO\(-\)derived species, such as nitrate, also produced in the presence of oxymyoglobin (23).

Although the association between respiratory rates, hydrogen peroxide production, and NO\(^+\) generation was apparent from these results, no quantitative correlation was demonstrated. Moreover, given the fact that mitochondria are endowed with a pool of GSNO\(^+\) and that its synthesis (25) and decay may produce oxygen radicals (26) at rates that may be significant, we sought to explore these processes using quantitative strategies.

Role of S-Nitrosoglutathione Metabolism in the Hydrogen Peroxide Generation by Mitochondria—The major mitochondrial thiol is constituted by glutathione (about 10 \(\mu\)M) (27). Glutathione is synthesized in the cytosol, imported to the mitochondria, and involved in a protective role against oxidative stress (28). Thiols react with NO\(^+\) or its metabolites, resulting in the formation of stable NO\(^+\) donors or nitrosothiols (29). Recent reports suggest that the formation of GSNO \(_{in vivo}\) may include the production of O\(_2\)\(^{\bullet}\) via the oxidation of the intermediate GSNO\(^{\bullet}\) (25) (Equations 1 and 2).

\[
\text{GS}^- + \text{NO}^+ \rightarrow [\text{GSNO}^\bullet] \quad \text{(Eq. 1)}
\]

\[
[\text{GSNO}^\bullet] + \text{O}_2 \rightarrow \text{GSNO} + \text{O}_2^\bullet \quad \text{(Eq. 2)}
\]

If this mechanism had been active in mitochondria, assuming oxygen is the only suitable electron acceptor for Equation 2, increases in NO\(^+\) production would have been followed by increases in the rates of O\(_2\)^\bullet and GSNO production. Studies were undertaken to explore the occurrence of this mechanism in mitochondria and to explore whether the increase in oxygen radical production observed in NO\(-\)producing mitochondria could be associated with the formation of GSNO. In addition, the decay of GSNO, which includes a complex chemistry of homolytic and heterolytic mechanisms (26, 30), could also result in the production of hydrogen peroxide. Toward this end, the rates of the production and decay of GSNO were evaluated in terms of their potential to contribute to the rate of hydrogen peroxide produced by mitochondria. The concentration of GSNO from intact mitochondria (analyzed and quantified by HPLC as
As described under “Experimental Procedures,” the steady-state reduction of the carriers of the respiratory chain was evaluated in intact, purified mitochondria (31, 32) and on the fact that certain inhibitors of the respiratory chain (those that increase the steady-state reduction level of electron carriers at the NADH dehydrogenase and ubiquinol-cytochrome oxidase (in percentage) was found within the experimental range of the inhibition of the oxygen consumption under identical conditions. This result agreed with those previously obtained in which changes in the respiratory rates correlated with those in cytochrome oxidase activity under identical conditions of NO production (7).

Experimental evidence for the association between the increased reduced state of the respiratory chain carriers and H2O2 production (8). The respiratory control ratio and phosphate to oxygen ratio number of these preparations were 4.7 and 2.1, respectively, assayed in the presence of 10 mM succinate and a 0.45 mM ADP in reaction buffer (0.225 M sucrose, 10 mM potassium phosphate, 5 mM MgCl2, 20 mM Hepes, pH 7.4). The absorption spectra were recorded using an SLM-Aminco DW-2C UV-visible spectrophotometer as described previously (33). The difference spectra of samples, which contained 1 mg of mitochondrial protein in reaction buffer, were recorded under the following conditions: 20 mM succinate (State 4; striped bar), succinate plus 0.5 mM ADP (State 3; white bar), succinate and 50 mM antimycin (State 4 plus antimycin; gray bar), and succinate and 0.15 mM L-arginine (State 4 plus L-arginine; black bar). The concentrations of each carrier were calculated using the following extinction coefficients: cytochromes b and c; 644–650 nm, 19 mM−1 cm−1; cytochromes c1, c550–560 nm, 19 mM−1 cm−1; and cytochromes c550–560 nm, 19 mM−1 cm−1. Oxygen and Nitrogen Radicals in Mitochondria

**Fig. 3.** Effect of NMMA on O2 uptake and H2O2 production by intact mitochondria. The H2O2 production (A) and O2 uptake (B) of intact, purified mitochondria supplemented with 0.1 mM L-Arg were measured in the presence of 5 mM glutamate/0.5 mM malate (closed circles) or 10 mM succinate (open circles). Other experimental conditions were described in the legend to Fig. 1 and under “Experimental Procedures.”

**TABLE I**

| Additions | −d[O2]/dt | +d[H2O2]/dt | +d[NO]/dt |
|-----------|-----------|-------------|-----------|
| NMMA      | 24.2 ± 0.4 | 0.11 ± 0.03 | 0.02 ± 0.01 |
| L-Arg     | 12.7 ± 0.2 | 0.24 ± 0.02 | 1.50 ± 0.05 |

*Not detectable. Significantly different (p < 0.01) by one-way analysis of variance (ANOVA).*
production in NO\(^{-}\)-producing mitochondria was provided by evaluating the steady-state reduction of the respiratory chain carriers and the rate of ROS production under a variety of conditions, including those that entailed changes in NO\(^{-}\) production (Fig. 6). A significant correlation was found between the reduction level of cytochrome b (Fig. 6) or other components of the respiratory chain (not shown) and the production of H\(_2\)O\(_2\) (Fig. 6) (\(r = 0.958\)), indicating that an increased reduction of these carriers under a variety of conditions (including those in which NO\(^{-}\) is produced) leads to an increased rate of ROS production. Based on our results, it seemed that the modulation of ROS production was accomplished through the reversible inhibition of the mitochondrial respiratory rate by NO\(^{-}\) at the cytochrome oxidase level. The transient decrease (or inhibition, depending on the concentration of NO\(^{-}\) and oxygen) in the respiratory rate leads to an increased steady-state reduction of the respiratory chain carriers, which in turn by reacting with oxygen resulted in the production of ROS. It is likely that cytochrome oxidase represents the crossover point in NO\(^{-}\)-producing mitochondria (Fig. 5), excluding a significant interaction of NO\(^{-}\} with other available components of the respiratory chain (cytochromes c or b) under these experimental conditions. However, if cytochrome oxidase were the crossover point in this process, it would have been fully reduced. This apparent discrepancy can be bridged by considering that the interaction of NO\(^{-}\} with cytochrome oxidase is complex, constituted by the reversible binding of NO\(^{-}\) to the heme a\(_3\) of cytochrome oxidase and by the reduction of NO\(^{-}\) to N\(_2\)O by this oxidase (7).

Considering the results presented in this study, it could be wrongly surmised that the effect of NO\(^{-}\} on the respiratory chain is not different from that of other xenobiotics used to enhance ROS production in mitochondria (e.g. NADH-supplemented mitochondria plus rotenone or FADH\(_2\)-supplemented mitochondria plus antimycin). Nitric oxide has two distinctive properties from the aforementioned inhibitors; it is endogenously produced (4–8), and it has a transient effect (5, 7). This latter property, based on its cytochrome c oxidase-mediated catabolism, allows a slow but continuous flow of electrons through the chain, even when a complete suppression of the O\(_2\) consumption has been accomplished. This type of modulation of the respiratory chain has the advantage of avoiding a complete reduction of the components of the respiratory chain and the subsequent burst of ROS when lower ratios of [NO\(^{-}\]/[O\(_2\)] are encountered.

**Conclusions**—The mitochondrial production of ROS has been considered as a side process of the normal oxidative metabolism, its rate ranging between two levels determined by the mitochondrial metabolic states, namely States 4 (maximum) and 3 (minimum) (2). This study demonstrated that the mitochondrial production of ROS is not limited to these two values and may exhibit a degree of values modulated by endogenous NO\(^{-}\}. Cellular conditions that would affect the availability of either l-Arg or other cofactors required for NOS activity would generate variable amounts of NO\(^{-}\) and O\(_2\)\(_{5}\}. Although further studies need to be performed to fully understand the implications of these observations, two different groups of reports may support the importance of our observations. First, some studies support the important role of manganese superoxide dismutase in a system that produces both O\(_2\) and NO\(^{-}\} in preventing the formation of the powerful oxidant peroxynitrite and the deleterious effect that the inactivation/lack of this enzyme may cause (20). Second, some studies examine the role of mitochondrial ROS, and that of endogenous agents that modulate their concentrations, as activators of intracellular signaling cascades involved in a variety of responses.

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