The reversible inhibitory effects of nitric oxide (NO) on mitochondrial cytochrome oxidase and \( \text{O}_2 \) uptake are dependent on intramitochondrial NO utilization. This study was aimed at establishing the mitochondrial pathways for NO utilization that regulate \( \text{O}_2 \) generation via reductive and oxidative reactions involving ubiquinol oxidation and peroxynitrite (ONOO–) formation. For this purpose, experimental models consisting of intact mitochondria, ubiquinone-depleted/reconstituted submitochondrial particles, and ONOO–-supplemented mitochondrial membranes were used.

The results obtained from these experimental approaches strongly suggest the occurrence of independent pathways for NO utilization in mitochondria, which effectively compete with the binding of NO to cytochrome oxidase, thereby releasing this inhibition and restoring \( \text{O}_2 \) uptake. The pathways for NO utilization are discussed in terms of the steady-state levels of NO and \( \text{O}_2 \) and estimated as a function of \( \text{O}_2 \) tension. These calculations indicate that mitochondrial NO decays primarily by pathways involving ONOO– formation and ubiquinol oxidation and, secondarily, by reversible binding to cytochrome oxidase.

In the early 1970s, it was recognized that isolated respiring mitochondria produce hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) at rates that depend on the redox state of the components of the respiratory chain and, consequently, on the mitochondrial metabolic state and the presence of inhibitors (1, 2). Mitochondrial production of \( \text{H}_2\text{O}_2 \) accounts for about 1% of the \( \text{O}_2 \) uptake under physiological conditions, according to evidence obtained from perfused rat liver and heart (3). Mitochondrial \( \text{H}_2\text{O}_2 \) is produced through the manganese-superoxide dismutase-catalyzed disproportionation of \( \text{O}_2 \) (4–6), which is vectorially generated into the mitochondrial matrix during ubisemiquinone autoxidation (4, 7, 8) and NADH-dehydrogenase activity (9). The relatively high rate of \( \text{O}_2 \) production in the mitochondrial inner membrane is in a functional relationship with the localization of superoxide dismutase in the mitochondrial matrix, which keeps a compartmentalized low steady-state concentration of \( \text{O}_2^\bullet\bullet \). Based on the rate of production of \( \text{O}_2^\bullet\bullet \), the content of manganese-superoxide dismutase in the mitochondrial matrix, and the corresponding second order rate constants, a \( [\text{O}_2^\bullet\bullet]_{\text{obs}} \) value of 0.5–1.0 \( \times 10^{-10} \) mol can be estimated (3, 10).

Nitric oxide (NO) produced by the endothelium elicits cellular physiological effects within a wide concentration range (10–9 to 10–5 M). The effects of NO on mitochondria—inhibition of cytochrome c oxidase (11–19), impairment of electron flow at the cytochrome bc1 region (17), and oxidation of ubiquinol (20, 21)—require progressively increasing concentrations of this species. NO regulates \( \text{O}_2 \) uptake and promotes \( \text{H}_2\text{O}_2 \) release by mitochondria (17, 22) (an effect also demonstrated in the isolated beating rat heart (23)); the increase in mitochondrial \( \text{H}_2\text{O}_2 \) formation may be understood as an antimycin-like effect of NO accomplished by its effective binding to the cytochrome bc1 segment (17).

The NO influx in the mitochondrial compartment is expected to affect the steady-state levels of \( \text{O}_2^\bullet\bullet \) due to the diffusion-controlled reaction between these species (24, 25) to yield peroxynitrite (ONOO–) (26). Three recently recognized facts add complexity to the mitochondrial interactions between \( \text{O}_2^\bullet\bullet \) and NO: first, NO inhibits succinate-cytochrome c reductase activity and increases \( \text{O}_2 \) production in submitochondrial particles, isolated mitochondria, and perfused rat heart (17, 23). Second, membrane-bound mitochondrial NOS generates NO at rates that are similar to the rates of mitochondrial \( \text{O}_2 \) production (27–29). Third, NO can be reduced to the nitroxyanion (NO•) by one-electron transfers from three reduced components of the mitochondrial respiratory chain: ubiquinol, cytochrome c, and cytochrome c oxidase (20, 30, 31).

The fine metabolic control of the intramitochondrial steady-state concentrations of NO—performed through a series of oxidative and reductive reactions involving \( \text{O}_2^\bullet\bullet \) ubiquinol, the cytochrome bc1 segment, and cytochrome c oxidase—is relevant to mitochondrial physiology with further implications for cell energy production. This study is aimed at establishing the mitochondrial pathways for NO utilization that regulate \( \text{O}_2 \) generation via reductive and oxidative reactions involving ubiquinol and ONOO–, respectively. For this purpose, experimental models consisting of ubiquinone-depleted/reconstituted submitochondrial particles and ONOO–-supplemented submitochondrial particles were used.

**MATERIALS AND METHODS**

Chemicals and Biochemicals—Cytochrome c, carboxylycyaide p-(tri-fluoromethoxy) phenylhydrazone, rhodamine 123, 5,5-dimethyl-1-pyrroline-N-oxide, \( \text{H}_2\text{O}_2 \), NaCN, myxothiazol, uric acid, fatty acid-free

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‡ The abbreviations used are: NOS, nitric oxide synthase; UQ0, ubiquinone-0; UQ10, ubiquinone-10; UQ10, ubiquinone-50.
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bovine serum albumin, NaNBH₄, ubiquinone-0 (UQ₀), (2,3-dimethoxy-6-methyl-1,4-benzoquinone), ubiquinone-10 (UQ₁₀) decylnubiquinone, and ubiquinone-50 (UQ₅₀) were from Sigma. DETAN0 was from Alexis Corp. (San Diego, CA). Ubiquinone reduction was carried out prior to the onset of the experiment upon addition of 10–20 µl of NaNBH₄ (20 mM solution) to 3 ml of 20 µM ubiquinones dissolved in water (UQ₀) or in ethanol (UQ₁₀). Ubiquinone solutions were purged with argon for 5 min in a flask sealed with a rubber septum; the excess of reductant was eliminated by adding HCl up to 80 mM. Nitric oxide solutions (1.2–1.8 mM) were obtained by bubbling NO gas (99.9% purity; AGA GAS Inc., Maumee, OH) in helium-purged water for 30 min at room temperature. NO solutions were stored at 4 °C. All other reagents were of analytical grade.

Isolation of Rat Liver Mitochondria—Excised livers (mean weight, 10 g) from adult Harlan Sprague-Dawley female rats (200–250 g) were placed in an ice-cold homogenization medium consisting of 0.23 M mannitol, 70 mM sucrose, 10 mM Tris-HCl, and 1 mM EDTA with 0.5% bovine serum albumin (pH 7.4). The tissue was finely minced and transferred to a motorized Teflon Potter-Elvehjem homogenizer (Thomas Scientific, Philadelphia, PA) and homogenized in 9 ml of cold homogenization medium per g of tissue. The homogenate was centrifuged at 700 x g for 10 min. The supernatant was centrifuged at 7000 x g for 10 min. The pellet was washed twice and resuspended in homogenization medium without bovine serum albumin at a protein concentration of 20 mg of mitochondrial protein/ml and lyophilized for use in experiments. NO solutions were prepared from frozen and thawed mitochondria (20 mg of mitochondrial protein/ml) disrupted by sonication for three 10-s periods at an output of 40 W using a model W-225 sonifier (Kontes, Vineland, NJ). Mitochondrial protein/ml was determined by the Lowry assay using bovine serum albumin as standard. Microsomes were prepared essentially as described previously (7) with minor modifications. Submitochondrial particles were resuspended in 0.15M KCl medium without bovine serum albumin at a protein concentration of 20 mg of mitochondrial protein/ml disrupted by sonication for three 10-s periods at an output of 40 W using a model W-225 sonifier (Kontes, Vineland, NJ). Mitochondrial protein/ml was determined by the Lowry assay using bovine serum albumin as standard.

Preparation of Submitochondrial Particles—Submitochondrial particles were prepared from frozen and thawed mitochondria (20 mg of mitochondrial protein/ml) disrupted by sonication for three 10-s periods at an output of 40 W using a model W-225 sonifier (Kontes, Vineland, NJ). Mitochondrial protein/ml was determined by the Lowry assay using bovine serum albumin as standard.

Mitochondrial Transmembrane Potential—Mitochondrial membrane potential was measured fluorometrically with ΔΨₘ and Δψₘ values of 503 and 527 nm, respectively (Hitachi Fluorometer model F2000, Hitachi Ltd., Tokyo, Japan) with excitation and emission wavelengths at 315 and 425 nm, respectively (34). The reaction mixture consisted of the respiratory medium described above supplemented with 8 mM succinate, 12 units/ml bovine serum albumin, 50 µM N-5-hydroxyphenyl acetic acid, and 0.1–0.5 mg of mitochondrial protein/ml.

Mitochondrial O₂ Production—O₂ production by liver submitochondrial particles was measured by superoxide dismutase-sensitive cytochrome c reduction at 550 nm (ε₅₅₀ = 21 m M–1 cm–1) in a reaction mixture consisting of respiratory medium supplemented with 1 mM succinate, 10 µM cytochrome c, 2.4 mM myxothiazol, 1 mM cyanide, and 0.1 mg of mitochondrial protein/ml in the absence of 10 mM superoxide dismutase (7).

Cytochrome Oxidase Activity—This activity was determined by monitoring the oxidation of 50 µM of reduced cytochrome c in a Hitachi U-3000 spectrophotometer at 550 nm (ε₅₅₀ = 21 m M–1 cm–1). Cytochrome c was reduced with potassium ascorbate followed by 24 h dialysis against 10 mM Na₂HPO₄/KH₂PO₄, pH 7.2. The rate of cytochrome c oxidation was determined as the pseudo-first order constant (k') and expressed as k'(min–1) mg protein–1.

Activity of Manganese-superoxide Dismutase—Manganese-superoxide dismutase activity was determined spectrophotometrically by inhibition of the rate of cytochrome c reduction (followed at 550 nm). The reaction mixture consisted of 20 µM cytochrome c, 0.5 mM xanthine, and xanthine oxidase in 50 mM potassium phosphate/0.1 mM EDTA, pH 7.8 (35). 1 mM MnCl₂ was used to inhibit copper-zinc-superoxide dismutase and cytochrome c oxidase activities.

Mitochondrial Protein Determination—Protein concentration was determined by the Lowry assay using bovine serum albumin as standard.

RESULTS

Effects of NO on Mitochondrial Respiration—NO utilization by liver mitochondria is evidenced by the first order decay of the NO signal with a t₁/₂ of 1.8 min (Fig. 1A, a). The initial rate of NO decay was linearly related to mitochondrial protein concentration (Fig. 1B, a), thus indicating the involvement of mitochondrial components in the pathway(s) for NO decay. From the plot in Fig. 1B, a, a rate of utilization of NO by mitochondria of 1 nmol/min/mg of protein may be calculated. NO elicited a complete and transient inhibition of mitochondrial O₂ uptake in state 3; respiration restarted when NO levels decreased to ~0.35 µM (Fig. 1A, b). Half-maximal inhibition of O₂ uptake in state 3 was observed at 0.17 µM NO (Fig. 1B, b). NO decreased the mitochondrial membrane potential as detected by changes in rhodamine fluorescence (33) (Fig. 1A, c); half-maximal inhibition of membrane potential was observed at about 0.15 µM NO (Fig. 1B, c).

Mitochondrial Pathways for NO Utilization—Fig. 2 shows the time courses of NO decay in the presence of rat liver sub mitochondrial particles under anaerobic and aerobic conditions. In anaerobiosis, NO metabolism is expected to be encompassed mainly by reductive pathways, i.e. the reduction of NO to the nitroxy anion (NO⁻), as follows.

\[ \text{NO + e}^- \rightarrow \text{NO}^- \]

REACTION 1
This reaction involves different electron donors. In this context, ubiquinol (Reaction 2) (20), cytochrome c (Reaction 3) (30), and cytochrome oxidase (Reaction 4) (31) were reported to facilitate the redox transition depicted in Reaction 1 (cyt c is cytochrome c).

\[
\text{NO} + \text{UQH}^2 \rightarrow \text{NO}^- + \text{H}^+ \\
\text{NO} + \text{cyt c}^{2+} \rightarrow \text{NO}^- + \text{cyt c}^{3+} \\
\text{NO} + \text{cyt a}^{2+} \rightarrow \text{NO}^- + \text{cyt a}^{3+}
\]

**REACTIONS 2–4**

Under anaerobic conditions, the rate of NO decay in the presence of submitochondrial particles supplemented with succinate and the inhibitor myxothiazol (which inhibits electron flow between cytochromes b and c) was \(-0.1 \text{ nmol/min/mg of protein (Fig. 2A, trace b)}\); this rate increased to \(-0.14 \text{ nmol/min/mg of protein in the absence of myxothiazol (Fig. 2A, trace c)}\). In the former instances, ubiquinol is the likely electron donor for Reaction 1; the rate constant of this reaction may be estimated as \(2.1 \times 10^8 \text{ M}^{-1} \text{s}^{-1}\) based on an ubiquinol content of \(-2 \text{ nmol/mg of mitochondrial protein under these experimental conditions (36)}\). In the latter instances—in absence of myxothiazol—reduction of cytochrome c (Reaction 3) and cytochrome oxidase (Reaction 4) also contribute to NO decay via a reductive pathway.

**FIG. 1. Effects of NO on mitochondrial respiration.** A, amperometric trace of NO decay following supplementation of a 2.5 \(\mu\text{M}\) solution of NO in respiratory medium (see under “Materials and Methods”) with mitochondria (1 mg of protein/ml), 6 mM malate/glutamate, and 0.1 mM ADP. b, time course of \(\text{O}_2\) consumption corresponding to 1 mg of mitochondrial protein/ml supplemented with 0.1 mM ADP and 6 mM malate/glutamate. c, fluorometric determination of mitochondrial membrane potential was assessed with a reaction mixture as in b above but with 0.25 mg of mitochondrial protein/ml and 6 mM succinate as substrate and in the presence of 0.2 \(\mu\text{M}\) rhodamine 123. Time of NO addition is indicated by the dotted line. A, dependence of NO decay rate on mitochondrial protein. Assay conditions were as in A, a with varying amounts of mitochondria. b, dependence of the rate of \(\text{O}_2\) uptake on NO concentration. Assay conditions were as in A, a with varying amounts of NO. c, dependence of mitochondrial membrane potential on NO concentration. Assay conditions were as in A, c in the presence of varying amounts of NO. Other assay conditions as described under “Materials and Methods.”

Under aerobic conditions and in the absence of submitochondrial particles, NO decay followed as a pseudo-first order process with a \(t_{1/2}\) of 6 min (Fig. 2B, trace a), which is consistent with a rate constant for the reaction of NO with \(\text{O}_2\) of \(1.6 \times 10^7 \text{ M}^{-2} \text{s}^{-1}\). (The \(t_{1/2}\) corresponding to a NO concentration of 0.1 \(\mu\text{M}\) in tissues with \(-20 \mu\text{M}\) \(\text{O}_2\) determined by this nonenzymatic reaction would be 8.6–23 h (37) and, hence, biologically negligible.) Succinate-supplemented submitochondrial particles decreased the \(t_{1/2}\) of NO decay to 1.7 min (Fig. 2B, trace b). This \(t_{1/2}\) value was decreased and increased by 0.4 \(\mu\text{M}\) \(\text{UQ}_0\) (Fig. 2B, trace c) and superoxide dismutase (Fig. 2B, trace d) (1.2 and 2.6 min, respectively). The effect elicited by superoxide dismutase suggests a role for \(\text{O}_2^-\) in the decay pathways of NO, as follows.

\[
\text{NO} + \text{O}_2^- \rightarrow \text{ONO}^2^-
\]

**REACTION 5**

The rates of NO utilization by rat liver submitochondrial particles under aerobic conditions were linearly dependent on NO concentrations in the 0.025–0.4 \(\mu\text{M}\) range and, consequently, followed first order kinetics (Fig. 3). From the plots in Fig. 3A, the rates of NO utilization by mitochondria under different conditions may be calculated: NO utilization by succinate-supplemented submitochondrial particles proceeded at rates of \(-0.3–0.5 \text{ nmol/min/mg of protein (when supplemented with 50–100 nM NO)}\). In the presence of superoxide dismutase, NO utilization by submitochondrial particles proceeded in a \(\text{O}_2^-\)-independent fashion at rates of \(-0.1–0.2 \text{ nmol/min/mg of protein} \). Conversely, submitochondrial particles supplemented with succinate and soluble ubiquinone to expand the reducible ubiquinone pool showed a higher NO utilization rate: 0.4–0.8 nmol/min/mg of protein. This rate was also inhibited by superoxide dismutase.

**-NO-induced Production of H_2O_2 and NO Utilization Are Dependent on Mitochondrial Ubiquinol Content—Early experiments with mitochondrial membranes depleted of endogenous...**
ubiquinone and reconstituted with variable amounts of ubiquinones showed a linear relationship between quinone content and H₂O₂ formation (7). Succinate-dependent H₂O₂ production in ubiquinone-depleted and ubiquinone-reconstituted membranes was linearly related to the ubiquinone content over a wide range of quinone levels (up to 26 nmol/mg of protein). Thus, ubiquinone autoxidation appears to be a major source of H₂O₂ production (via O₂ disproportionation) under conditions that involve inhibition of electron transport between cytochromes b and c (e.g. in the presence of antimycin A or myxothiazol) (7). The roles of nitric oxide and ubiquinol content in mitochondrial membranes in the production of H₂O₂ were assessed with two experimental models: ubiquinone-depleted submitochondrial particles in the absence of inhibitors of the electron transport chain (e.g. myxothiazol) and submitochondrial particles with an expanded ubiquinol pool in the presence of myxothiazol.

In the absence of myxothiazol, succinate-supplemented submitochondrial particles show negligible production of H₂O₂ (Fig. 4A); the addition of -NO slightly enhances H₂O₂ production under these conditions. Reconstitution of these mitochondrial membranes with variable amounts of ubiquinone results in increasing H₂O₂ production with increasing -NO concentrations up to 0.25 μM. Beyond this -NO concentration, only slight increases of H₂O₂ formation were observed (Fig. 4A). Both -NO utilization and H₂O₂ production by succinate-supplemented, ubiquinone-depleted submitochondrial particles were linearly dependent on the amount of ubiquinone (UQ₁₀) added (Fig. 4B).

These experiments suggest that, first, ubiquinone is essential for the production of H₂O₂ by mitochondrial membranes and, second, that -NO (in the absence of myxothiazol) may elicit two effects in a concentration-dependent manner: on the one hand, -NO inhibits electron flow at the cytochrome bc₃ segment (17) (analogous to the effects of antimycin A and myxothiazol) and, on the other hand, -NO oxidizes ubiquinol (Reaction 2) (20), thus triggering ubisemiquinone autoxidation (Reaction 6; \( k₆ = 8 \times 10^9 \text{M}^{-1} \text{s}^{-1} \)) and, thereby, H₂O₂ production (Reaction 7). The notions shown in the following reactions,

\[
\begin{align*}
\text{UQ}^* &\rightarrow \text{UQ} + \text{O}_2^- \\
\text{O}_2^- + \text{H}_2\text{O} &\rightarrow \text{H}_2\text{O}_2 + \text{O}_2
\end{align*}
\]

are strengthened by experiments carried out with submitochondrial particles with an intact ubiquinone content and in which the ubiquinone pool was expanded upon addition of Q₀ or Q₂ (Fig. 4C): under these conditions and with a fixed concentration of -NO, the production of H₂O₂ increases with increasing supplementation of the particles with either quinone. Intact submitochondrial particles supplemented with UQ₀ show a succinate-ubiquinone reductase activity of about 70 nmol/min/mg of protein in the presence of myxothiazol (Fig. 4D); addition of -NO results in oxidation of the formed UQ₀H₂ at a rate of ~24 nmol/min/mg of protein (Fig. 4D). The latter rate reflects only partially the ubiquinol oxidation by -NO because of concomitant reduction of the quinone by succinate dehydrogenase.

Taken together, these experimental models suggest that H₂O₂ formation by mitochondrial membranes requires ubisemiquinone autoxidation accomplished in a sequential manner by, on the one hand, increasing the ubiquinol pool by impairing electron flow at the bc₃ segment and, on the other hand, increasing ubiquinol oxidation by -NO (Reaction 2).
performed with intact mitochondria (Fig. 1A, b). NO also inhibits temporarily O₂ consumption by ubiquinone-depleted/reconstituted submitochondrial particles. The role of ubiquinol in the NO-inhibitable respiration was assessed with three experimental designs, as follows.

(a) O₂ uptake was measured in ubiquinone-depleted/reconstituted submitochondrial particles (Fig. 5A). In these instances, the period of inhibition of O₂ uptake elicited by NO (until respiration was restored) was decreased with increasing concentrations of UQ₁₀ incorporated into the membranes (UQ₁₀ reincorporated was measured as succinate-reducible ubiquinone, thus indicating that reincorporation took place at specific sites in the respiratory chain critical for electron transfer). Half-maximal inhibitory effect was obtained with 15 nmol of UQ₁₀ per mg of protein.

(b) Cytochrome oxidase activity was measured in ubiquinone-depleted/reconstituted submitochondrial particles with a myxothiazol-inhibited electron flow and supplemented with reduced cytochrome c (Fig. 5B). Under these conditions, the activity of cytochrome oxidase was measured as cytochrome c oxidation. As described above, the period of inhibition of cytochrome c oxidation exerted by nitric oxide was decreased with increasing concentrations of UQ₁₀ incorporated into the mitochondrial membranes.

(c) Cytochrome oxidase activity was measured in submitochondrial particles with an increased ubiquinol pool (accomplished by supplementation with increasing concentrations of UQ₀ and UQ₂) and in the presence of myxothiazol (Fig. 5C). Similar to the results described above, this resulted in a decrease of the time of inhibition of cytochrome oxidase activity by NO. Half-maximal inhibitory effects were obtained with 20 and 50 μM UQ₀ and UQ₂, respectively.

These experimental approaches strongly suggest the occurrence of independent pathways for NO utilization in mitochondria, which effectively compete with the binding of NO to cytochrome oxidase, thereby releasing this inhibition and restoring O₂ uptake. Hence, in these experimental models, the ubiquinone-dependent decrease of the temporary inhibition of O₂ uptake elicited by NO may be understood as a competition between cytochrome oxidase (Reaction 4), ubiquinol (Reaction 2), and O₂⁻ (Reaction 5) for NO, regardless of whether the succinate oxidase activity was inhibited or not by myxothiazol. This is illustrated by the decrease in the inhibition time of cytochrome oxidase by increasing ubiquinone concentrations under conditions in which electron transfer is blocked by myxothiazol (Fig. 5B).

The significance of Reaction 5 for the mitochondrial pathways of NO utilization is suggested by the increase in cytochrome oxidase inhibition time by superoxide dismutase (Fig. 5D) in experiments in which the ubiquinone pool of submitochondrial particles was augmented by addition of UQ₀. An alternative reductive pathway for NO may be its reaction with reduced cytochrome c (Reaction 3) (30), although the contribution of this reaction to the intramitochondrial decay rate of NO is uncertain, because it probably occurs on the C phase of the inner mitochondrial membrane during diffusion of NO to or from the cytosol.

The results of the H₂O₂ generation shown in Fig. 6, the mitochondrial steady-state level of O₂⁻ can be calculated, considering (a) that O₂⁻ is the stoichiometric precursor of H₂O₂, (b) that the main reactions of O₂⁻ utilization are the superoxide dismutase-catalyzed disproportionation (k = 1.9 – 2.3 × 10⁻⁸ M⁻¹ s⁻¹) and the reaction with NO (k₅ = 1.9 × 10¹⁰ M⁻¹ s⁻¹), and (c) the concentration of manganese superoxide dismutase in the mitochondrial matrix (0.3 × 10⁻⁵ M) (3, 10).

\[ \frac{d[O_2^-]}{dt} = k[SOD][O_2^-] + k[NO][O_2^-] \quad (Eq. 1) \]

The steady-state level of O₂⁻ ([O₂⁻]ₘ) in the mitochondrial matrix calculated from the above equation is in the 10⁻¹¹ M range.
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**FIG. 6. Effects of NO on the H$_2$O$_2$ production of submitochondrial particles.** Measured values of H$_2$O$_2$ production rate (●) by submitochondrial particles (0.12 mg of protein/ml) supplemented with 6 mM succinate and different amounts of NO. The steady-state concentration of O$_2$ (▲) and the rate of production of ONOO$^-$ (○) for the corresponding data points of H$_2$O$_2$ formation were calculated as described in the text.

**FIG. 7. Influence of NO on HO$_2$ formation by succinate-supplemented submitochondrial particles.** Assay conditions: A, submitochondrial particles (2 mg of protein/ml) supplemented with 6 mM succinate and 80 mM 5,5-dimethyl-1-pyrroline-N-oxide. B, as in A in the presence of 5 μM NO. C, as in B but with 20 μM NO. Instrument settings: receiver gain, 2 × 106; microwave power, 20 mW; microwave frequency, 9.81 GHz; modulation amplitude, 2.420 G; time constant, 1.3 s.

range, a value obtained from a rate of O$_2^+$ production of $-1.2 \times 10^{-6}$ M s$^{-1}$ (calculated from a rate of H$_2$O$_2$ production of $-0.13$ nmol/min/mg of protein at 2 μM NO in Fig. 6 and a volume for 1 mg of mitochondrial protein of 3.6 μl (39, 40)).

$$[O_2]_{ss} = -d[O_2]/dt/[(SOD) + k([NO])$$  
(Eq. 2)

$$[O_2]_{ss} = 1.2 \times 10^{-6} \text{ ms}^{-1/2} \times 10^{10} \text{ M}^{-1} \text{s}^{-1} \times [0.3 \times 10^{-5} \text{ M}] + 1.9 \times 10^{15} \text{ M}^{-1} \text{s}^{-1} \times [2 \times 10^{-6} \text{ M}]$$

(Eq. 3)

$$[O_2]_{ss} = 2.7 \times 10^{-11} \text{ M}$$

(Eq. 4)

The above equations may be used to estimate the rate of ONOO$^-$ production by mitochondrial membranes as it is affected by varying concentrations of NO. At the same concentration of NO utilized for the above calculations ($2 \times 10^{-6}$ M) and considering a $k_5$ value for the reaction of $1.9 \times 10^{10}$ M$^{-1}$ s$^{-1}$ (41), the following equations hold true.

$$+d[\text{ONOO}^-]/dt = k_5[O_2][\text{NO}]$$

(Eq. 5)

$$+d[\text{ONOO}^-]/dt = 1.9 \times 10^{10} \text{ M}^{-1} \text{s}^{-1} \times [2.7 \times 10^{-11} \text{ M}] [2 \times 10^{-6} \text{ M}]$$

(Eq. 6)

$$+d[\text{ONOO}^-]/dt = 1.02 \times 10^{6} \text{ M} \text{s}^{-1}$$

(Eq. 7)

Both the steady-state level of O$_2^+$ and the rate of ONOO$^-$ formation were plotted for the individual experimental points for H$_2$O$_2$ production in Fig. 6. The rate of production of ONOO$^-$ is slow at low NO concentrations and when the rate of formation of H$_2$O$_2$ is at its maximum; conversely, as the rate of H$_2$O$_2$ decreases with increasing concentrations of NO, the rate of ONOO$^-$ generation increases. This may strengthen the conclusions drawn from the data in Fig. 7, in which HO$_2^-$ generation—derived from H$_2$O$_2$ scission—by mitochondrial occurs at low NO concentrations and it switches to ONOO$^-$ at high NO levels. It may also be surmised that the concentration of NO in the reaction mixture determines the steady-state level of O$_2^+$.

**Peroxynitrite as a Source of O$_2^+$**—Supplement of submitochondrial particles with succinate in the presence of myxothiazol results in increased levels of ubiquinol. The further addition of low concentrations (in the 0.25–2 μM range) of ONOO$^-$ elicited a production of O$_2^+$ (Fig. 8A), consistent with an oxidation of the membrane-bound ubiquinol to the corresponding ubisemiquinone (Reaction 8) and its subsequent autoxidation to yield O$_2^+$ (Reaction 6). The yield for this reaction was 0.5 O$_2^+$ generated per ONOO$^-$ added (Fig. 8A).

$$\text{UQH}_2^- + \text{ONOO}^- \rightarrow \text{UQ}^*_2^- + \text{NO}_2^- + \text{HO}^-$$

**REACTION 8**

Reaction 8 is expected to proceed freely given the reduction potentials of the redox couples involved ($E(UQ_2^+/UQH^-) = -0.19$ V; $E(\text{NO}_2^-/\text{NO}_2^-\text{H}^-) = +1.4$ V) (42, 43).

Additional indirect evidence for the reactivity of peroxynitrite toward ubiquinol may be surmised from the experiments in Fig. 8B: amperometric measurement of NO in submitochondrial particles supplemented with DETANO (a steady source of NO) revealed steady-state levels of this species of $-0.5$ μM. Addition of succinate resulted in a decrease of NO steady-state levels to $-0.2$ μM, whereas the subsequent supplementation of the reaction mixture with superoxide dismutase restored NO concentration to the initial level (Fig. 8B). The latter effect suggests NO utilization via O$_2^+$ (as depicted in Reaction 5) and, hence, formation of ONOO$^-$. Considering the inside-out character of the submitochondrial particles (in which the M side of the mitochondrial inner membrane is exposed to the reaction medium and the electrode), determination of NO concentration under the conditions of Fig. 8B may model the matrix of intact mitochondria in a manner that NO electrode is monitoring an expanded mitochondrial matrix.

**DISCUSSION**

NO, produced both by the NOS of the endothelial cells (44) and the mitochondrial NOS in the inner mitochondrial membrane (27–29), plays key roles as intracellular and intercellular messenger in the regulation of tissue O$_2$ uptake and energy production. Endothelium-produced NO reversibly inhibits cytochrome c oxidase activity and cell O$_2$ uptake, an action that allows O$_2$ molecules to further diffuse in the tissue and that decreases the steepness of the O$_2$ gradient in the normoxia/anoxia transition (17, 23). The active production of NO by the mitochondrial NOS in the inner mitochondrial membrane (at
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FIG. 8. ONOO\(^-\)-dependent \(O_2^\cdot\) generation by submitochondrial particles. A, \(O_2^\cdot\) production rate and \(O_2^\cdot\) production by succinate-supplemented submitochondrial particles (6 mM succinate and 0.12 mg of protein/ml) in the presence of 2.4 \(\mu\)M myxothiazol with different concentrations of ONOO\(^-\). B, amperometric trace of the effect of submitochondrial particles and superoxide dismutase on the steady-state level of \(O_2\). Assay conditions: NO levels were reached by decomposition of DETANO in respiration medium. Arrow indicates the addition of submitochondrial particles (0.12 mg of protein/ml) and succinate (6 mM). Where indicated, 2 \(\mu\)M superoxide dismutase (SOD) was added.

rates of 0.3–1.5 nmol/min/mg of protein, in both states 3 and 4 (29) sets an additional feed back mechanism for the kinetic control of mitochondrial electron transfer and \(O_2\) uptake.

These multiple regulatory actions require specialized pathways of \(\cdot\)NO metabolism, considering that the rate of the non-enzymatic reaction of \(\cdot\)NO with \(O_2\) is negligible at the tissue \(pO_2\). Most of the experimental data in this study are consistent with the notion that the reaction of \(\cdot\)NO with \(O_2^\cdot\) (24, 25) (Reaction 5) describes a major route of mitochondrial \(\cdot\)NO utilization under aerobic conditions. Evaluation of the significance of \(O_2^\cdot\) in the decay pathway of \(\cdot\)NO in mitochondria requires consideration of (a) the effects elicited by \(\cdot\)NO on the mitochondrial respiratory chain, (b) the mechanisms for mitochondrial generation of \(O_2^\cdot\) in the presence of \(\cdot\)NO, (c) the steady-state levels of \(\cdot\)NO and \(O_2^\cdot\) in mitochondria, and (d) the modulation of the \(\cdot\)NO utilization pathways in mitochondria by oxygen tensions.

(a) Cytochrome spectra are consistent with a multiple inhibition of the mitochondrial respiratory chain in the presence of \(\cdot\)NO, predominantly involving binding of these species to cytochrome oxidase and the cytochrome bc\(_1\) segment. The reaction of \(\cdot\)NO with the former probably involves two binding sites (14); ferrocytochrome \(a_4\) and Cu\(_{\beta}\), the latter having a lower affinity for \(\cdot\)NO. The inhibition of NADH- and succinate-cytochrome c reductase activities by relatively high concentration of NO (1.2 \(\times\) 10\(^{-6}\) M) (17) suggests that, in addition to the reported effect on cytochrome oxidase (11), there is a second \(\cdot\)NO-sensitive site in the common pathway for both reductases of the electron transfer chain. \(\cdot\)NO elicits cytochrome \(b\) reduction in the presence of succinate, whereas cytochromes \(a\_a\) and \(c\) remain oxidized; this effect indicates inhibition of electron transfer at the \(O_2^\cdot\) side of cytochrome \(b\) (17) in a manner that resembles the effects exerted by antimycin A and myxothiazol.

(b) The above sequence builds a situation in which \(O_2^\cdot\) and \(H_2O_2\) may be generated, probably involving autooxidizable components on the electron donor side of cytochrome \(b\). Consistent with this notion, \(\cdot\)NO-mediated inhibition of mitochondrial electron transfer resulted in an enhancement of \(O_2^\cdot\) production by submitochondrial particles (Fig. 6) as well as of \(H_2O_2\) in isolated mitochondria (17) and in perfused heart (23). This effect is transient because removal of \(\cdot\)NO upon its reaction with \(O_2^\cdot\) (producing ONOO\(^-\) (Reaction 5)) would “release” cytochromes from the inhibitory effects. Considering the rates of \(\cdot\)NO and \(O_2^\cdot\) production under physiological conditions and the short half-life of ONOO\(^-\) (less than 1 s), the intramitochondrial production of this species should remain at relatively low rates (see calculations below).

The \(\cdot\)NO-induced inhibition cytochrome bc\(_1\) segment increases the steady-state level of reduced ubiquinone, thereby amplifying the potential of the reaction between UQH\(_2\) and \(\cdot\)NO (Reaction 2) (20); a \(k_2\) value of \(-2 \times 10^3\) M\(^{-1}\) s\(^{-1}\) may be calculated from the UQH\(_2\) content in rat mitochondrial membranes.

(c) The steady-state level of \(\cdot\)NO in the mitochondrial matrix may be estimated at 5 \(\times\) 10\(^{-8}\) M, a mean value derived from the \(\cdot\)NO level measured in isolated rat diaphragm (2 \(\times\) 10\(^{-8}\) M) (45), that measured in perfused rat heart stimulated by bradykinin (1 \(\times\) 10\(^{-7}\)) (23), and that calculated for rat liver mitochondria (5 \(\times\) 10\(^{-8}\) M) (29). The steady-state level of \(O_2^\cdot\) in the mitochondrial matrix has been calculated as \(-10^{-12}\) M (3).

(d) The \(\cdot\)NO utilization pathways in mitochondria are expected to be modulated by \(O_2\) tensions and to be an expression of the above steady-state concentrations. Under aerobic conditions, these steady-state values and the \(k_2\) value permit to calculate the actual rate of ONOO\(^-\) formation by mitochondria from the differential equation of Reaction 5 in a manner analogous to that shown for the experimental conditions of Fig. 6.\(^2\) Based on these steady-state levels, an actual rate of ONOO\(^-\) formation in mitochondria of 9.5 \(\times\) 10\(^{-8}\) M s\(^{-1}\) can be calculated. The contribution of cytochrome oxidase to nitric oxide metabolism under aerobic conditions may be inferred from the slow first order (decay of the cyt \(a_3\)\(^2+\)-\(\cdot\)NO compound (Reaction 4; \(k_4\) = 0.13 s\(^{-1}\) (46) and assuming a 10% of cytochrome oxidase as cyt \(a_3\)\(^2+\)-\(\cdot\)NO compound (\(-0.06\) nmol/mg of protein); this results in a rate of \(\cdot\)NO metabolism via cytochrome oxidase of \(-0.8 \times 10^{-8}\) M s\(^{-1}\).

At very low \(O_2\) tensions, it has been proposed that mitochondria catalyzed \(\cdot\)NO breakdown by two separate mechanisms presumably involving reductive reactions (15). One of these reductive pathways is sensitive to azide and cyanide and apparently involves reduction of \(\cdot\)NO at cytochrome oxidase site (Reaction 4) (15). The other reductive pathway may be represented by the interaction of \(\cdot\)NO with ubiquinol as illustrated in

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\(^2\)The rate of formation of ONOO\(^-\) calculated under “Dual Effect of \(\cdot\)NO on \(H_2O_2\) Production by Mitochondrial Membranes” under “Results” was based on the experimental conditions of Fig. 6, involving a concentration of \(\cdot\)NO of 2 \(\times\) 10\(^{-6}\) M.
Nitric Oxide Utilization by Mitochondria


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The Regulation of Mitochondrial Oxygen Uptake by Redox Reactions Involving Nitric Oxide and Ubiquinol
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