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

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The reversible inhibitory effects of nitric oxide (·NO) on mitochondrial cytochrome oxidase and O_2 uptake are dependent on intramitochondrial ·NO utilization. This study was aimed at establishing the mitochondrial pathways for ·NO utilization that regulate $O_2^{\cdot-}$ 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 O_2 uptake. The pathways for ·NO utilization are discussed in terms of the steady-state levels of ·NO and $O_2^{\cdot-}$ and estimated as a function of 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 (H_2O_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 H_2O_2 accounts for about 1% of the O_2 uptake under physiological conditions, according to evidence obtained from perfused rat liver and heart (3). Mitochondrial H_2O_2 is produced through the manganese-superoxide dismutase-catalyzed disproportionation of $O_2^{\cdot-}$ (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 $O_2^{\cdot-}$ 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 $O_2^{\cdot-}$ ($[O_2^{\cdot-}]_{ss}$). Based on the rate of production of $O_2^{\cdot-}$, the content of manganese-superoxide dismutase in the mitochondrial matrix, and the corresponding second order rate constants, a $[O_2^{\cdot-}]_{ss}$ value of 0.5–1.0 $\times$ 10^{-10} M 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 bc_1 region (17), and oxidation of ubiquinol (20, 21)—require progressively increasing concentrations of this species. ·NO regulates O_2 uptake and promotes H_2O_2 release by mitochondria (17, 22) (an effect also demonstrated in the isolated beating rat heart (23)); the increase in mitochondrial H_2O_2 formation may be understood as an antimycin-like effect of ·NO accomplished by its effective binding to the cytochrome bc_1 segment (17).

The ·NO influx in the mitochondrial compartment is expected to affect the steady-state levels of $O_2^{\cdot-}$ 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 $O_2^{\cdot-}$ and ·NO: first, ·NO inhibits succinate-cytochrome *c* reductase activity and increases $O_2^{\cdot-}$ production in submitochondrial particles, isolated mitochondria, and perfused rat heart (17, 23). Second, membrane-bound mitochondrial NOS[1] generates ·NO at rates that are similar to the rates of mitochondrial $O_2^{\cdot-}$ production (27–29). Third, ·NO can be reduced to the nitroxyl anion (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 $O_2^{\cdot-}$, ubiquinol, the cytochrome bc_1 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 $O_2^{\cdot-}$ 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*, carbonylcyanide *p*-(trifluoromethoxy) phenylhydrazone, rhodamine 123, 5,5-dimethyl-1-pyrroline-*N*-oxide, H_2O_2, NaCN, myxothiazol, uric acid, fatty acid-free

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[1] The abbreviations used are: NOS, nitric oxide synthase; UQ_0, ubiquinone-0; UQ_2, ubiquinone-10; UQ_{10}, ubiquinone-50.

bovine serum albumin, $NaBH_4$, ubiquinone-0 (UQ_0) (2,3-dimethoxy-6-methyl-1,4-benzoquinone), ubiquinone-10 (UQ_2) (decylubiquinone), and ubiquinone-50 (UQ_{10}) were from Sigma. DETANO 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 $NaBH_4$ (20 mM solution in 0.1 N NaOH) to 3 ml of 20 mM quinones dissolved in either water (UQ_0) or in ethanol (UQ_2); 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 stocked 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-Elvejhem homogenizer (Thomas Scientific, Philadelphia, PA) and homogenized in 9 ml of cold homogenization medium per g of tissue. The homogenate was centrifuged at $700 \times g$ for 10 min. The supernatant was centrifuged at $7000 \times g$ for 10 min. The pellet was washed twice and resuspended in homogenization medium without bovine serum albumin at a protein concentration of 20 mg/ml. All procedures were performed at 4° C.

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 with 30-s intervals at an output of 40 W using a model W-225 sonifier (Heat Systems/Ultrasonics, Chicago, IL). Submitochondrial particles were washed twice and resuspended in the homogenization medium described above. All procedures were carried out at 4 °C.

Preparation of Ubiquinone-depleted Submitochondrial Particles—Ubiquinone-depleted and reconstituted submitochondrial particles were prepared essentially as described previously (7) with minor modifications. Submitochondrial particles were resuspended in 0.15 M KCl at a concentration of 20 mg of protein/ml and lyophilized for 9 h to completely dehydrate the samples. Mitochondrial ubiquinone was removed by suspending the lyophilizate in *n*-pentane by gentle homogenizations. Extracted ubiquinone was 4–5 nmol/mg of protein. Reincorporation of ubiquinone into submitochondrial particles was accomplished by resuspending the ubiquinone-depleted particles in a small volume of *n*-pentane (1–2 ml) containing UQ_{10} at a concentration of 50–100 nmol/mg of protein; the suspension was shaken in an iced bath for 30 min. The particles were centrifuged, dried by evaporation for 1 h, and stored. UQ_{10} content of ubiquinone-reconstituted particles (5–40 nmol/mg of mitochondrial protein) was determined by high pressure liquid chromatography with electrochemical detection using an amperometric detector (Bioanalytical Systems, West Lafayatte, IN).

Mitochondrial Respiratory Activities and Respiratory Control—O_2 uptake was determined polarographically with a Clark-type electrode in an air-saturated (0.24 mM O_2) reaction medium consisting of 0.23 M mannitol, 70 mM sucrose, 30 mM Tris-HCl, 4 mM $MgCl_2$, 5 mM Na_2HPO_4/KH_2PO_4, and 1 mM EDTA, pH 7.4 (respiration medium) and containing 1–2 mg of mitochondrial protein/ml. O_2 uptake was determined with 6 mM malate/glutamate as substrates, in the presence (state 3) and absence (state 4) of phosphate acceptor (0.2 mM ADP). O_2 uptake was expressed in ng-at O/min/mg of protein. ADP:O ratios were calculated as the ratio of nmol of ADP_{added}/ng-at $O_{utilized}$ during state 3 respiration (32). Respiratory control and ADP:O values of isolated mitochondria were 6–9 and 2.9–3.2, respectively.

Mitochondrial Transmembrane Potential—Mitochondrial membrane potential was measured fluorometrically with λ_{exc} and λ_{em} values of 503 and 527 nm, respectively (Hitachi Fluorometer model F200, Hitachi Ltd., Tokyo, Japan). The reaction mixture consisted of 0.25 mg of mitochondrial protein/ml in the respiratory medium described above supplemented with 0.2 μM rhodamine 123 (33). Complete polarization of the membrane was achieved by addition of 6 mM succinate and depolarization by pulses of ·NO.

Electron Paramagnetic Resonance—Electron paramagnetic resonance spectra were recorded on a Bruker ECS 106 equipped with a TM 8810 microwave cavity (Bruker Analytik GmbH, Rheinstetten, Germany). Measurements were carried out at room temperature at a microwave frequency of 9.80 GHz and 100 kHz field modulation. Other instrument settings were as described in the figure legends.

Determination of Nitric Oxide—·NO was determined amperometrically with an electrode ISO-NO (World Precision Instruments, Sarasota, FL) in 3 ml of respiration medium (see above) with electromagnetic stirring at 30° C. The ·NO electrode was calibrated daily with $NaNO_2$ in acid medium (0.1 M H_2SO_4-KI) to generate known concentrations of ·NO.

Absorption Spectroscopy—Absorption spectra of ubiquinones were obtained with a Hitachi U-3000 spectrophotometer (Hitachi Ltd., Tokyo, Japan) from submitochondrial particles supplemented with 40 μM UQ_0 and 1 mM KCN in 50 mM H_2NaPO_4/HNa_2PO_4, pH 7.4. For the anaerobic experiments, the reaction medium was deoxygenated by bubbling argon during 30 min and placed in 1 ml quartz cuvettes. The cuvettes were sealed with a rubber septum and an aliquot of a ·NO solution was injected into the cuvette with a gas-tight syringe (final concentration, 30 μM ·NO). The spectral changes were recorded in the UV region; increase in absorbance at 266 nm indicated the oxidation of ubiquinol to ubiquinone (ϵ_{266} = 14.2 mM^{-1} cm^{-1}).

Mitochondrial H_2O_2 Production—H_2O_2 production was continuously monitored by the horseradish peroxidase/*p*-hydroxyphenyl acetic acid assay in the Hitachi F-2000 spectrofluorometer (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 horseradish peroxidase, 50 μM *p*-hydroxyphenyl acetic acid, and 0.1–0.5 mg of mitochondrial protein/ml.

Mitochondrial O_2^- Production—O_2^- production by liver submitochondrial particles was measured by superoxide dismutase-sensitive cytochrome *c* reduction at 550 nm (ϵ_{550} = 21 mM^{-1} cm^{-1}) in a reaction mixture consisting of respiratory medium supplemented with, 1 mM succinate, 10 μM cytochrome *c*, 2.4 μM myxothiazol, 1 mM cyanide, and 0.1 mg of mitochondrial protein/ml in the presence or absence of 10 nM 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 (ϵ_{550} = 21 mM^{-1} cm^{-1}). Cytochrome *c* was reduced with potassium ascorbate followed by 24 h dialysis against 10 mM Na_2HPO_4/KH_2PO_4, pH 7.2. The rate of cytochrome *c* oxidation was determined as the pseudo-first order constant reaction (k') and expressed as $k'(min^{-1})$ mg $protein^{-1}$.

Activity of Manganese-superoxide Dismutase—Manganese-superoxide dismutase 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 NaCN 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.

Data Analysis—Data shown in figures express mean values from duplicate determinations.

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 $t_{1/2}$ of 1.8 min (Fig. 1*A, a*). The initial rate of ·NO decay was linearly related to mitochondrial protein concentration (Fig. 1*B, a*), thus indicating the involvement of mitochondrial components in the pathway(s) for ·NO decay. From the plot in Fig. 1*B, 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 O_2 uptake in state 3; respiration restarted when ·NO levels decreased to ~0.35 μM (Fig. 1*A, b*). Half-maximal inhibition of O_2 uptake in state 3 was observed at 0.17 μM ·NO (Fig. 1*B, b*). ·NO decreased the mitochondrial membrane potential as detected by changes in rhodamine fluorescence (33) (Fig. 1*A, c*); half-maximal inhibition of membrane potential was observed at about 0.15 μM ·NO (Fig. 1*B, c*).

Mitochondrial Pathways for ·NO Utilization—Fig. 2 shows the time courses of ·NO decay in the presence of rat liver submitochondrial 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 nitroxyl anion (NO^-), as follows.

$$\cdot NO + e^- \rightarrow NO^-$$

REACTION 1

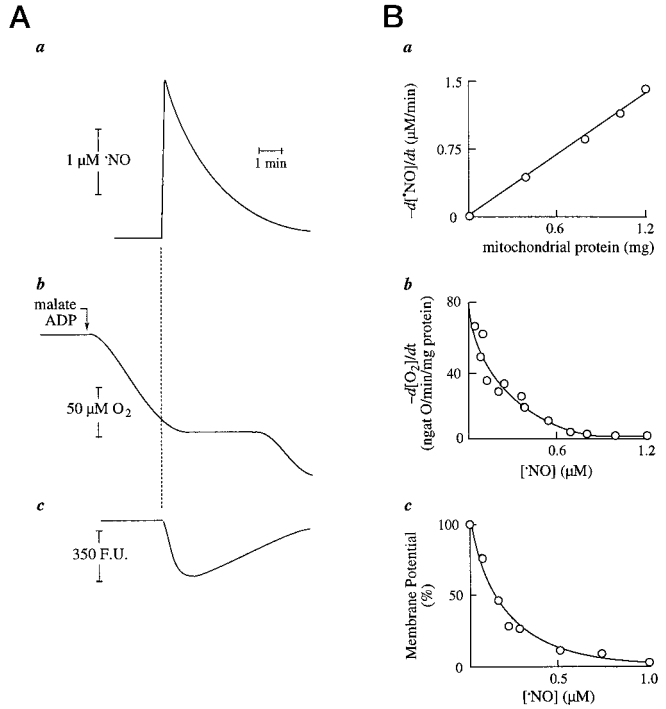

FIG. 1. **Effects of ·NO on mitochondrial respiration.** *A, a,* amperometric trace of ·NO decay following supplementation of a 2.5 μM solution of ·NO in respiratory medium (see under "Materials and Methods") with mitochondria (1 mg of protein/ml), 6 mM malate/glutamate, and 0.1 mM ADP. *b,* time course of 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 μM rhodamine 123. Time of ·NO addition is indicated by the *dotted line*. *B, 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 O_2 uptake on ·NO concentration. Assay conditions were as in *A, b* 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."

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*).

$$\cdot NO + UQH^- \rightarrow NO^- + H^+$$

$$\cdot NO + \text{cyt } c^{2+} \rightarrow NO^- + \text{cyt } c^{3+}$$

$$\cdot NO + \text{cyt } a_3^{2+} \rightarrow NO^- + \text{cyt } a_3^{3+}$$

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 nmol/min/mg of protein (Fig. 2*A, trace b*); this rate increased to ~0.14 nmol/min/mg of protein in the absence of myxothiazol (Fig. 2*A, 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 2.1×10^3 M^{-1} s^{-1} based on an ubiquinol content of ~2 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.

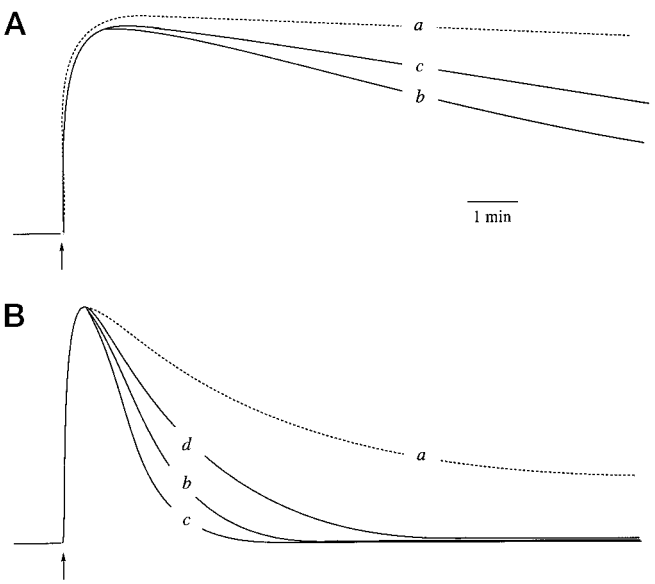

FIG. 2. **Time course of the ·NO decay under anaerobic and aerobic conditions.** *A,* amperometric traces of ·NO decay (initial concentration, 0.4 μM) in an anaerobic solution of respiration medium in the absence of submitochondrial particles (*a*) and the presence of submitochondrial particles (0.1 mg of protein/ml) and 6 mM succinate (*b*). *c,* as in *b* in the presence of 2.4 μM myxothiazol. *B,* amperometric traces of ·NO decay in aerobiosis: *a,* no further additions; *b,* submitochondrial particles (0.1 mg of protein/ml) plus 6 mM succinate; *c,* as in *b* plus 2 μM UQ_0; *d,* as in *b* plus 2 μM superoxide dismutase. The addition of 0.4 μM ·NO is indicated by the *arrows*.

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. 2*B, trace a*), which is consistent with a rate constant for the reaction of ·NO with O_2 of 1.6×10^7 M^{-2} s^{-1}. (The $t_{1/2}$ corresponding to a ·NO concentration of 0.1 μM in tissues with ~20 μM 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. 2*B, trace b*). This $t_{1/2}$ value was decreased and increased by UQ_0 (Fig. 2*B, trace c*) and superoxide dismutase (Fig. 2*B, trace d*) (1.2 and 2.6 min, respectively). The effect elicited by superoxide dismutase suggests a role for O_2^- in the decay pathways of ·NO, as follows.

$$\cdot NO + O_2^- \rightarrow ONOO^-$$

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 μM range and, consequently, followed first order kinetics (Fig. 3). From the plots in Fig. 3*A,* 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 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 O_2^--independent fashion at rates of 0.1–0.2 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

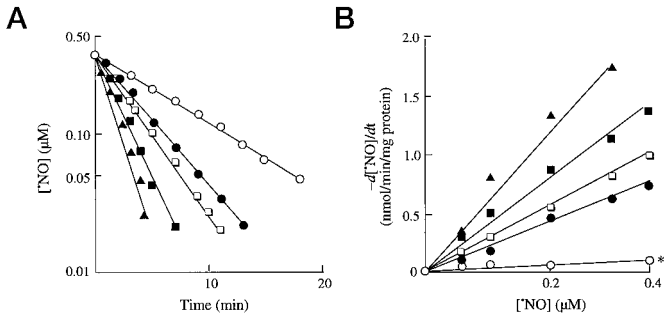

FIG. 3. **·NO utilization by submitochondrial particles and dependence on ·NO concentration.** *A,* semilogarithmic plot of ·NO concentration *versus* time. A solution of ·NO in air-saturated respiratory medium without further addition (○), with submitochondrial particles (●), with submitochondrial particles plus succinate (■), with submitochondrial particles plus succinate and UQ_0 (▲), and with submitochondrial particles plus succinate and superoxide dismutase (□). Concentrations of reactants were as in Fig. 2*B*. *B,* dependence of the rate of ·NO utilization on ·NO concentration. Assay conditions were as in *A* with varying amounts of ·NO. *, the units for these values (○) are μM/min (assay carried out in the absence of submitochondrial particles).

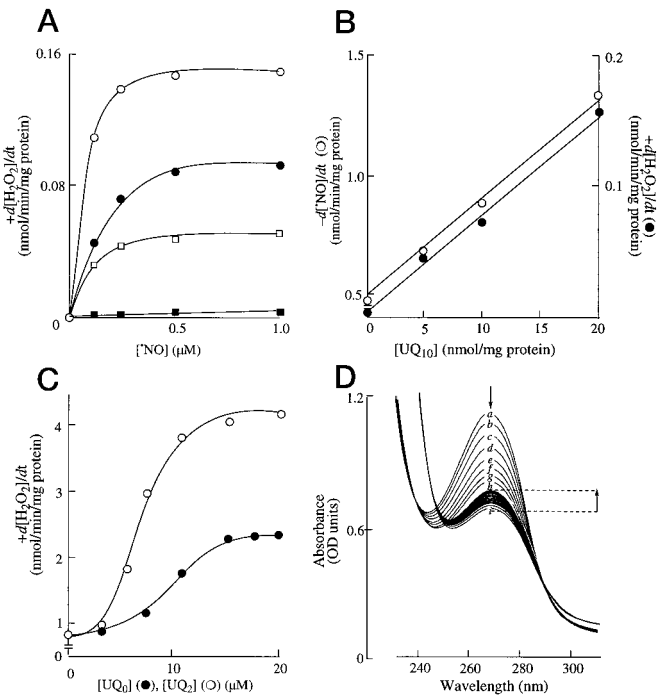

FIG. 4. **Dependence of H_2O_2 production and ·NO utilization by submitochondrial particles on ubiquinone concentration.** *A,* ·NO-induced H_2O_2 production by ubiquinone-depleted submitochondrial particles. Assay conditions: ubiquinone-depleted or ubiquinone-restored submitochondrial particles in respiration medium were supplemented with 6 mM succinate and varying amounts of ·NO. ■, ubiquinone-depleted submitochondrial particles. □, ●, and ○, ubiquinone-depleted submitochondrial particles supplemented with 5-, 10-, and 20 nmol of UQ_{10}/mg of protein, respectively. *B,* dependence of the rates of ·NO utilization and H_2O_2 production on UQ_{10} content. Assay conditions: ubiquinone-depleted/reconstituted submitochondrial particles in respiration medium were supplemented with 6 mM succinate and 1 μM ·NO and different amounts of UQ_{10}. *C,* effect of ubiquinones on H_2O_2 formation by submitochondrial particles. Assay conditions: 0.12 mg of submitochondrial particle protein/ml in respiration medium were supplemented with 5 μM ·NO and different amounts of UQ_0 or UQ_2. *D,* succinate-ubiquinone reductase activity of submitochondrial particles. The absorption spectrum was obtained in anaerobiosis from a reaction mixture consisting of submitochondrial particles (0.025 mg of protein/ml) in respiration medium supplemented with 45 μM UQ_0 and 2.4 μM myxothiazol (spectrum *a*). The reaction was initiated upon addition of 6 mM succinate, which resulted in a reduction of ubiquinone (decrease of absorbance) (*downward arrow*; *spectra b–i*). Addition of ·NO (40 μM) under the conditions of *spectrum i* resulted in ubiquinol oxidation (*upward arrow*).

ubiquinone and reconstituted with variable amounts of ubiquinones showed a linear relationship between quinone content and H_2O_2 formation (7). Succinate-dependent H_2O_2 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 H_2O_2 production (via O_2^- 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 H_2O_2 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 H_2O_2 (Fig. 4*A*); the addition of ·NO slightly enhances H_2O_2 production under these conditions. Reconstitution of these mitochondrial membranes with variable amounts of ubiquinone results in increasing H_2O_2 production with increasing ·NO concentrations up to 0.25 μM. Beyond this ·NO concentration, only slight increases of H_2O_2 formation were observed (Fig. 4*A*). Both ·NO utilization and H_2O_2 production by succinate-supplemented, ubiquinone-depleted submitochondrial particles were linearly dependent on the amount of ubiquinone (UQ_{10}) added (Fig. 4*B*).

These experiments suggest that, first, ubiquinone is essential for the production of H_2O_2 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 bc_1 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; $k_6 = 8 \times 10^3\ M^{-1}\ s^{-1}$) and, thereby, H_2O_2 production (Reaction 7). The notions shown in the following reactions,

$$UQ^{\cdot-}{}_2 + O_2 \rightarrow UQ + O_2^-$$

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

REACTIONS 6 AND 7

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 Q_0 or Q_2 (Fig. 4*C*): under these conditions and with a fixed concentration of ·NO, the production of H_2O_2 increases with increasing supplementation of the particles with either quinone.

Intact submitochondrial particles supplemented with UQ_0 show a succinate-ubiquinone reductase activity of about 70 nmol/min/mg of protein in the presence of myxothiazol (Fig. 4*D*); addition of ·NO results in oxidation of the formed UQ_0H_2 at a rate of ~24 nmol/min/mg of protein (Fig. 4*D*). 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 H_2O_2 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 bc_1 segment and, on the other hand, increasing ubiquinol oxidation by ·NO (Reaction 2).

·NO-Inhibition of Mitochondrial O_2 Uptake and Its Dependence on Ubiquinol Concentration—As shown with experiments

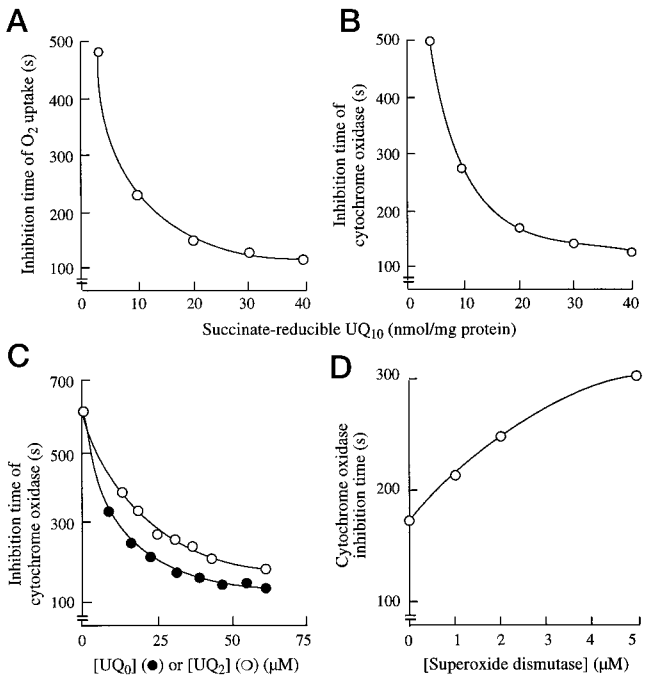

FIG. 5. **Effect of ubiquinone on the temporary inhibition of O_2 uptake and cytochrome oxidase activity by ·NO.** *A,* effect of ubiquinone on ·NO-mediated inhibition of O_2 consumption by ubiquinone-depleted/reconstituted submitochondrial particles. Assay conditions: ubiquinone-depleted submitochondrial particles (0.15 mg of protein/ml) in respiration medium were supplemented with 6 mM succinate, 20 μM ·NO, and varying amounts of UQ_{10}. *B,* effect of ubiquinone on ·NO-mediated cytochrome *c* oxidation by ubiquinone-depleted reconstituted submitochondrial particles. Assay conditions: ubiquinone-depleted submitochondrial particles (0.15 mg of protein/ml) in respiration medium were supplemented with 2.4 μM myxothiazol, 6 mM succinate, 50 μM reduced cytochrome *c*, 20 μM ·NO, and varying amounts of UQ_{10}. *C,* ·NO-mediated inhibition of cytochrome oxidase activity in submitochondrial particles with an increased ubiquinol pool. Assay conditions: submitochondrial particles (0.12 mg of protein) in respiration medium were supplemented with 2.4 μM myxothiazol, 6 mM succinate, 20 μM ·NO, 50 μM reduced cytochrome *c*, and varying amounts of either UQ_0 or UQ_2. *D,* effect of superoxide dismutase on the ·NO-mediated inhibition of cytochrome oxidase. Assay conditions: submitochondrial particles (0.12 mg of protein/ml) in respiration medium were supplemented with 20 μM UQ_0, 20 μM ·NO, 50 μM reduced cytochrome *c*, and varying amounts of superoxide dismutase.

performed with intact mitochondria (Fig. 1*A, b*), ·NO also inhibits temporarily O_2 consumption by submitochondrial particles. The role of ubiquinol in the ·NO-inhibitable respiration was assessed with three experimental designs, as follows.

(*a*) O_2 uptake was measured in ubiquinone-depleted/reconstituted submitochondrial particles (Fig. 5*A*). In these instances, the period of inhibition of O_2 uptake elicited by ·NO (until respiration was restored) was decreased with increasing concentrations of UQ_{10} incorporated into the membranes (UQ_{10} 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 UQ_{10}/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. 5*B*). 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 UQ_{10} 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 UQ_0 and UQ_2) and in the presence of myxothiazol (Fig. 5*C*). 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 UQ_0 and UQ_2, 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 O_2 uptake. Hence, in these experimental models, the ubiquinone-dependent decrease of the temporary inhibition of O_2 uptake elicited by ·NO may be understood as a competition between cytochrome oxidase (Reaction 4), ubiquinol (Reaction 2), and $O_2^{\cdot-}$ (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. 5*B*).

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. 5*D*) in experiments in which the ubiquinone pool of submitochondrial particles was augmented by addition of UQ_0. 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.

Dual effect of ·NO on H_2O_2 Production by Mitochondrial Membranes—·NO elicited a biphasic effect on the production of H_2O_2 by submitochondrial particles: at concentrations below 3 μM, ·NO increased H_2O_2 production, whereas at concentrations above this value, in the range of 3–20 μM, ·NO decreased mitochondrial H_2O_2 generation (Fig. 6). Likewise, ·NO exerted a biphasic effect on HO· formation by submitochondrial particles (Fig. 7): ·NO, at a concentration of 2.5 μM, increased the generation of the α-hydroxyethyl adduct of 5,5-dimethyl-1-pyrroline-*N*-oxide by succinate-supplemented submitochondrial particles in a fashion similar to antimycin A (38). (The α-hydroxy-ethyl adduct originates from the $HO^{\cdot}{}_2^-$mediated H abstraction of ethanol, thereby furnishing evidence for the occurrence of this species; the formation of HO· at this concentration of ·NO was further confirmed by the inhibitory effect of catalase (not shown)). At a concentration of 20 μM, ·NO abolished the electron paramagnetic resonance signal (Fig. 7*C*), probably due to the removal of $O_2^{\cdot-}$ by excess of ·NO with concomitant formation of $ONOO^-$ (Reaction 5). Hence, it may be expected that the production of HO· by mitochondrial membranes switches to that of $ONOO^-$ upon increasing concentrations of ·NO.

From the rates of H_2O_2 generation shown in Fig. 6, the mitochondrial steady-state level of $O_2^{\cdot-}$ can be calculated, considering (*a*) that $O_2^{\cdot-}$ is the stoichiometric precursor of H_2O_2, (*b*) that the main reactions of $O_2^{\cdot-}$ utilization are the superoxide dismutase-catalyzed disproportionation ($k = 1.9–2.3 \times 10^9$ M^{-1} s^{-1}) and the reaction with ·NO ($k_5 = 1.9 \times 10^{10}$ M^{-1} s^{-1}), and (*c*) the concentration of manganese superoxide dismutase in the mitochondrial matrix (0.3×10^{-5} M) (3, 10).

$$+d[O_2^{\cdot-}]/dt = k[\text{SOD}][O_2^{\cdot-}] + k[\cdot\text{NO}][O_2^{\cdot-}] \qquad \text{(Eq. 1)}$$

The steady-state level of $O_2^{\cdot-}$ ($[O_2^{\cdot-}]_{ss}$) in the mitochondrial matrix calculated from the above equation is in the 10^{-11} M

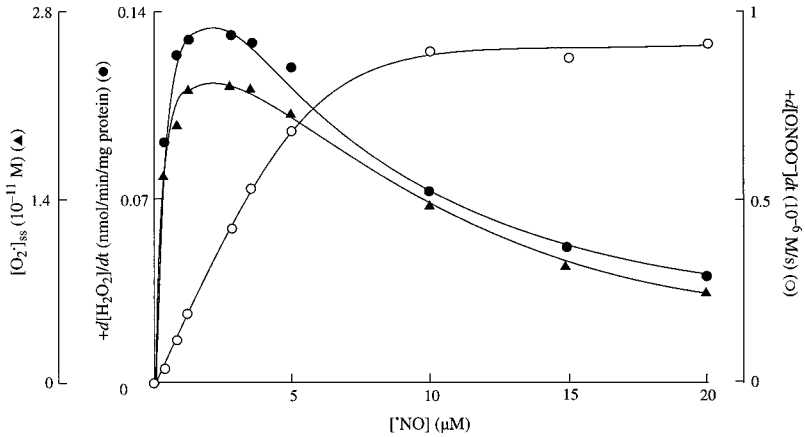

FIG. 6. **Effects of ·NO on the H_2O_2 production of submitochondrial particles.** Measured values of H_2O_2 production rate (●) by submitochondrial particles (0.12 mg of protein/ml) supplemented with 6 mM succinate and different amounts of ·NO. The steady-state concentration of $O_2^{\cdot-}$ (▲) and the rate of production of $ONOO^-$ (○) for the corresponding data points of H_2O_2 formation were calculated as described in the text.

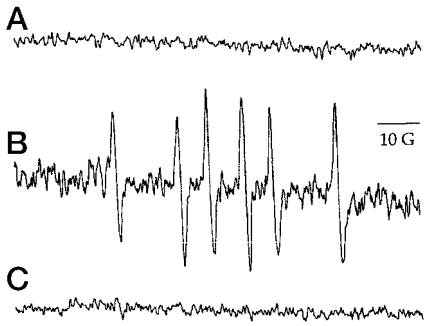

FIG. 7. **Influence of ·NO on HO· formation by succinate-supplemented submitochondrial particles.** Assay conditions: *A,* submitochondrial particles (2 mg of protein/ml) supplemented with 6 mM succinate and 80 mM 5,5-dimethyl-1-pyrroline-*N*-oxide. *B,* as in *A* in the presence of 5 μM ·NO. *C,* as 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 $O_2^{\cdot-}$ production of ~1.2×10^{-6} M s^{-1} (calculated from a rate of H_2O_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)).

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

$$[O_2^{\cdot-}]_{ss} = 1.2 \times 10^{-6}\ \text{M}s^{-1}/2.3 \times 10^{9}\ \text{M}^{-1}s^{-1}[0.3 \times 10^{-5}\ \text{M}] + 1.9 \times 10^{10}\ \text{M}^{-1}s^{-1}[2 \times 10^{-6}\ \text{M}] \quad \text{(Eq. 3)}$$

$$[O_2^{\cdot-}]_{ss} = 2.7 \times 10^{-11}\ \text{M} \quad \text{(Eq. 4)}$$

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 (2×10^{-6} M) and considering a k_5 value for the reaction of 1.9×10^{10} M^{-1} s^{-1} (41), the following equations hold true.

$$+d[ONOO^-]/dt = k_5[O_2^{\cdot-}][\cdot NO] \quad \text{(Eq. 5)}$$

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

$$+d[ONOO^-]/dt = 1.02 \times 10^{-6}\ \text{M}\ s^{-1} \quad \text{(Eq. 7)}$$

Both the steady-state level of $O_2^{\cdot-}$ and the rate of $ONOO^-$ formation were plotted for the individual experimental points for H_2O_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_2O_2 is at its maximum; conversely, as the rate of H_2O_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· generation—derived from H_2O_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 $O_2^{\cdot-}$.

Peroxynitrite as a Source of $O_2^{\cdot-}$—Supplementation 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 $O_2^{\cdot-}$ (Fig. 8*A*), consistent with an oxidation of the membrane-bound ubiquinol to the corresponding ubisemiquinone (Reaction 8) and its subsequent autoxidation to yield $O_2^{\cdot-}$ (Reaction 6). The yield for this reaction was 0.5 $O_2^{\cdot-}$ generated per $ONOO^-$ added (Fig. 8*A*).

$$UQH^- + ONOO^- \rightarrow UQ^{\cdot-}{}_2 + \cdot NO_2 + HO^- \quad \text{REACTION 8}$$

Reaction 8 is expected to proceed freely given the reduction potentials of the redox couples involved ($E(UQ_2^{\cdot-}/UQH^-) \sim 0.19$ V; $E(ONOO^-/NO_2\cdot,HO^-) = +1.4$ V) (42, 43).

Additional indirect evidence for the reactivity of peroxynitrite toward ubiquinol may be surmised from the experiments in Fig. 8*B*: 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. 8*B*). The latter effect suggests ·NO utilization via $O_2^{\cdot-}$ (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. 8*B* 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

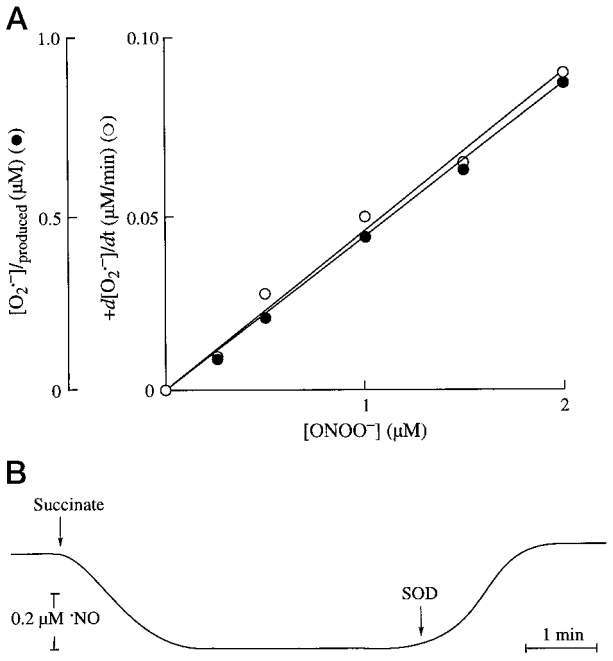

FIG. 8. **$ONOO^-$-dependent $O_2^{\cdot-}$ generation by submitochondrial particles.** *A,* $O_2^{\cdot-}$ production rate and $[O_2^{\cdot-}]_{produced}$ by succinate-supplemented submitochondrial particles (6 mM succinate and 0.12 mg of protein/ml) in the presence of 2.4 μM myxothiazol with different concentrations of $ONOO^-$. *B,* amperometric trace of the effect of submitochondrial particles and superoxide dismutase on the steady-state level of ·NO. 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 μM 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 ·NO metabolism, considering that the rate of the nonenzymatic reaction of ·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 ·NO with $O_2^{\cdot-}$ (24, 25) (Reaction 5) describes a major route of mitochondrial ·NO utilization under aerobic conditions. Evaluation of the significance of $O_2^{\cdot-}$ in the decay pathway of ·NO in mitochondria requires consideration of (*a*) the effects elicited by ·NO on the mitochondrial respiratory chain, (*b*) the mechanisms for mitochondrial generation of $O_2^{\cdot-}$ in the presence of ·NO, (*c*) the steady-state levels of ·NO and $O_2^{\cdot-}$ in mitochondria, and (*d*) the modulation of the ·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 ·NO, predominantly involving binding of these species to cytochrome oxidase and the cytochrome bc_1 segment. The reaction of ·NO with the former probably involves two binding sites (14): ferrocytochrome a_3 and Cu_B^+, the latter having a lower affinity for ·NO. The inhibition of NADH- and succinate-cytochrome *c* reductase activities by relatively high concentration of ·NO (1.2×10^{-6} M) (17) suggests that, in addition to the reported effect on cytochrome oxidase (11), there is a second ·NO-sensitive site in the common pathway for both reductases of the electron transfer chain. ·NO elicits cytochrome *b* reduction in the presence of succinate, whereas cytochromes aa_3 and *c* remain oxidized; this effect indicates inhibition of electron transfer at the O_2 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 autoxidizable components on the electron donor side of cytochrome *b*. Consistent with this notion, ·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 ·NO upon its reaction with $O_2^{\cdot-}$ (producing $ONOO^-$ (Reaction 5)) would "release" cytochromes from the inhibitory effects. Considering the rates of ·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).

TABLE I
Mitochondrial steady-state levels of $O_2^{\cdot-}$ and ·NO and the pathways of ·NO utilization

Steady-state levels	
$[O_2^{\cdot-}]_{ss}$	1.0×10^{-10} M
$[\cdot NO]_{ss}$	5.0×10^{-8} M
$O_2^{\cdot-}$ and H_2O_2 generation rates	
$+d[O_2^{\cdot-}]/dt$	6.9×10^{-6} M s^{-1}
$+d[H_2O_2]/dt$	3.4×10^{-6} M s^{-1}
·NO utilization pathways	
$+d[ONOO^-]/dt$	9.5×10^{-8} M s^{-1}
$-d[UQH^-]/dt$	3.1×10^{-8} M s^{-1}
$+d[\text{cyt } c^{2+} - \cdot NO]/dt$	0.8×10^{-8} M s^{-1}

The ·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 ·NO (Reaction 2) (20); a k_2 value of $\sim 2 \times 10^3$ M^{-1} s^{-1} may be calculated from the UQ_9H_2 content in rat mitochondrial membranes.

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

(*d*) The ·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_5 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 9.5×10^{-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+}-·NO compound (Reaction 4; $k_4 = 0.13$ s^{-1}) (46) and assuming a 10% of cytochrome oxidase as cyt a_3^{2+}-·NO compound (~0.06 nmol/mg of protein); this results in a rate of ·NO metabolism via cytochrome oxidase of $\sim 0.8 \times 10^{-8}$ M s^{-1}.

At very low O_2 tensions, it has been proposed that mitochondria catalyzed ·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 ·NO at cytochrome oxidase site (Reaction 4) (15). The other reductive pathway may be represented by the interaction of ·NO with ubiquinol as illustrated in

[2] The rate of formation of $ONOO^-$ calculated under "Dual Effect of ·NO on H_2O_2 Production by Mitochondrial Membranes" under "Results" was based on the experimental conditions of Fig. 6, involving a concentration of ·NO of 2×10^{-6} M.

Reaction 2 (20). The contribution of Reaction 4 (and possibly Reaction 3) to ·NO reductive decay is relatively low, and it may be inferred to be ~0.04 nmol ·NO/min/mg of protein (Fig. 2*A*), although this reaction has not been confirmed with purified cytochrome oxidase (47).

It is also of interest to establish the fate of $O_2^{\cdot-}$ vectorially released into the mitochondrial matrix during ubisemiquinone autoxidation (Reaction 6). Both ·NO (Reaction 2) and $ONOO^-$ (Reaction 8) may contribute to the build up of ubisemiquinone and, via Reaction 6, to $O_2^{\cdot-}$ formation. $O_2^{\cdot-}$ thus provides a regulatory mechanism to remove ·NO and, thereby, the inhibition of cytochrome oxidase. $O_2^{\cdot-}$ may be reduced to H_2O_2 (Reaction 7) by the action of manganese-superoxide dismutase or converted to $ONOO^-$ upon its reaction with ·NO (Reaction 5). Based on the above steady-state levels and considering that $O_2^{\cdot-}$ is the stoichiometric precursor of H_2O_2, the rate of formation of the latter may be calculated as 3.45×10^{-7} M s^{-1}. The rate of formation of $ONOO^-$ may be estimated as 9.5×10^{-8} M s^{-1}, as discussed above. Hence, a value of 0.28 may be obtained for the ratio $+d[ONOO^-]/dt/+d[H_2O_2]/dt$, thereby suggesting that $ONOO^-$ accounts for ~15% of $O_2^{\cdot-}$ generated by mitochondria, with the remaining 85% yielding H_2O_2 as final product. These values (Table I) are expected to change with the steady-state levels of nitric oxide: for example, under conditions of endothelial NOS activation, ·NO levels reach 100 nM in bradykinin-stimulated perfused heart (23) and 470 nM in the diaphragm of lipopolysaccharide-shocked rats (45). Under these conditions, $ONOO^-$ formation may account for as much as 28 and 72%, respectively, of $O_2^{\cdot-}$ utilization.

Table I summarizes the mitochondrial steady-state concentrations for ·NO and $O_2^{\cdot-}$, as well as the relative significance of mitochondrial utilization pathways for ·NO under normoxic conditions. It may be surmised that in rat liver mitochondria, ·NO is largely metabolized through the oxidative formation of $ONOO^-$ (9.5×10^{-8} M s^{-1}; 59%) and oxidation of ubiquinol (3.1×10^{-8} M s^{-1}; 36%) and, secondarily, through reduction by cytochrome oxidase and cytochrome *c* (0.8×10^{-8} M s^{-1}; 5%).

The control of mitochondrial functions depends on two variables, ·NO and O_2 tension, each one acting on concentration range and gradients and intersecting at critical points. The steady-state level of O_2 in mammalian organs is 5–25 μM O_2, and, especially in heart, 3–8 μM (48). These O_2 levels are in a range in which physiological concentrations of ·NO (50–100 nM) define values for the $[O_2]/[\cdot NO]$ ratios of 50–100 that experimentally produce 35–15% inhibition of mitochondrial respiration (22); under conditions of severe cytochrome oxidase inhibition, the feedback self-regulatory mechanism provided by the initial ubiquinol/·NO interaction appears crucial to maintain mitochondrial function. $ONOO^-$ seems to be a major product of ·NO utilization in aerobiosis: whether $ONOO^-$, formed under these conditions, would elicit oxidative damage in mitochondria would depend on its site of generation (49), its interception by matrix GSH (50), and, hypothetically, its reduction by the peroxynitrite reductase activity of mitochondrial glutathione peroxidase (51).

REFERENCES

1. Boveris, A., Oshino, N., and Chance, B. (1972) *Biochem. J.* **128,** 617–630
2. Boveris, A., and Chance, B. (1973) *Biochem. J.* **134,** 707–716
3. Boveris, A., and Cadenas, E. (1997) in *Oxygen, Gene Expression and Cellular Function* (Mazzaro, D., and Clerch, L., eds) pp. 1–25, Marcel Dekker Inc., New York
4. Boveris, A., and Cadenas, E. (1975) *FEBS Lett.* **54,** 311–314
5. Dionisi, O., Galeotti, T., Terranova, T., and Azzi, A. (1975) *Biochim. Biophys. Acta* **403,** 292–301
6. Chance, B., Sies, H., and Boveris, A. (1979) *Physiol. Rev.* **59,** 527–605
7. Boveris, A., Cadenas, E., and Stoppani, A. O. M. (1976) *Biochem. J.* **156,** 435–444
8. Cadenas, E., Boveris, A., Ragan, C. I., and Stoppani, A. O. M. (1977) *Arch. Biochem. Biophys* **180,** 248–257
9. Turrens, J. F., and Boveris, A. (1980) *Biochem. J.* **191,** 421–427
10. Tyler, D. D. (1975) *Biochem. J.* **147,** 493–504
11. Cleeter, M. W. J., Cooper, J. M., Darley-Usmar, V. M., Moncada, S., and Schapira, A. H. V. (1994) *FEBS Lett.* **345,** 50–54
12. Brown, G. C., and Copper, C. E. (1994) *FEBS Lett.* **356,** 295–298
13. Brown, G. C. (1995) *FEBS Lett.* **369,** 136–139
14. Torres, J., Darley-Usmar, V., and Wilson, M. T. (1995) *Biochem. J.* **312,** 169–173
15. Borutaité, V., and Brown, G. C. (1996) *J. Biochem.* **315,** 295–299
16. Cassina, A., and Radi, R. (1996) *Arch. Biochem. Biophys.* **328,** 309–316
17. Poderoso, J. J., Carreras, M. C., Lisdero, C., Riobó, N., Schöpfer, F., and Boveris, A. (1996) *Arch. Biochem. Biophys.* **328,** 85–92
18. Brown, G. C. (1997) *Mol. Cell Biochem.* **174,** 189–192
19. Koivisto, A., Matthias, A., Bronnkov, G., and Nedergaard, J. (1997) *FEBS Lett.* **417,** 75–80
20. Poderoso, J. J., Carreras, M. C., Schöpfer, F., Lisdero, C., Riobó, N., Giulivi, C., Boveris, A. D., Boveris, A., and Cadenas, E. (1999) *Free Radical Biol. Med.* **26,** 925–935
21. Cadenas, E., Poderoso, J. J., Antunes, F., and Boveris, A. (1999) in *Antioxidant Food Supplements in Human Health* (Packer, L., Hiramatsu, M., and Yoshikawa, T., eds) pp. 143–163, Academic Press, San Diego
22. Boveris, A., Costa, L. E., Cadenas, E., and Poderoso, J. J. (1999) *Methods Enzymol.* **301,** 188–198
23. Poderoso, J. J., Peralta, J. G., Lisdero, C. L., Carreras, M. C., Radisic, M., Schöpfer, F., Cadenas, E., and Boveris, A. (1998) *Am. J. Physiol.* **274,** C112–C119
24. Hui, R. E., and Padmaja, S. (1993) *Free Rad. Res. Commun.* **18,** 195–199
25. Beckman, J. S. (1990) *Nature* **345,** 27–28
26. Packer, M. A., Porteous, C. M., and Murphy, M. P. (1996) *Biochem. Mol. Biol. Int.* **40,** 527–534
27. Ghafourifar, P., and Richter, C. (1997) *FEBS Lett.* **418,** 291–296
28. Giulivi, C., Poderoso, J. J., and Boveris, A. (1998) *J. Biol. Chem.* **273,** 11038–11043
29. Giulivi, C. (1998) *Biochem. J.* **332,** 673–679
30. Sharpe, M. A., and Cooper, C. E. (1998) *J. Biochem.* **332,** 9–19
31. Zhao, X., Sampath, V., and Caughey, W. S. (1995) *Biochem. Biophys. Res. Commun.* **212,** 1054–1060
32. Estabrook, R. W. (1967) *Methods Enzymol.* **10,** 41–47
33. Takehara, Y., Kanno, T., Yoshioka, T., Inoue, M., and Utsumi, K. (1995) *Arch. Biochem. Biophys.* **323,** 27–32
34. Carreras, M. C., Poderoso, J. J., Cadenas, E., and Boveris, A. (1996) *Methods Enzymol.* **269,** 65–75
35. McCord, J. M., and Fridovich, I. (1969) *J. Biol. Chem.* **244,** 6049–6055
36. Boveris, A., and Stoppani, A. O. M. (1970) *Arch. Biochem. Biophys.* **141,** 641–655
37. Ford, P. C., Wink, D. A., and Stanbury, D. M. (1993) *FEBS Lett.* **326,** 1–3
38. Giulivi, C., Boveris, A., and Cadenas, E. (1995) *Arch. Biochem. Biophys.* **316,** 909–916
39. Beavis, A. D., Brannan, R. D., and Garlid, K. D. (1985) *J. Biol. Chem.* **260,** 13424–13433
40. Antunes, F., Salvador, A., Marinho, H. S., Alves, R., and Pinto, R. E. (1996) *Free Radical Biol. Med.* **21,** 917–943
41. Kissner, R., Nauser, T., Bugnon, P., Lye, P. G., and Koppenol, W. H. (1997) *Chem. Res. Toxicol.* **10,** 1285–1292
42. Brandt, U. (1996) *FEBS Lett.* **387,** 1–6
43. Koppenol, W. H. (1996) *Methods Enzymol.* **268,** 7–12
44. Ignarro, L., Buga, G. M., Wood, K. S., Byrns, R. E., and Chaudhuri, G. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 9265–9269
45. Boczkowski, J., Lisdero, C. L., Lanone, S., Carreras, M. C., Boveris, A., Aubier, M., and Poderoso, J. J. (1999) *FASEB J.* **13,** 1637–1647
46. Giuffre, A., Sarti, P., D'Itri, E., Buse, G., Soulimane, T., and Brunori, M. A. D. (1996) *J. Biol. Chem.* **271,** 33404–33408
47. Stubauer, G., Giuffré, A., Brunori, M., and Sarti, P. A. D. (1998) *Biochem. Biophys. Res. Commun.* **245,** 459–465
48. Wittenberg, B. A., and Wittenberg, J. B. (1989) *Annu. Rev. Physiol.* **51,** 857–878
49. Lizasoain, I., Moro, M. A., Knowles, R. G., Darley-Usmar, V., and Moncada, S. (1996) *Biochem. J.* **314,** 877–880
50. Augusto, O., Gatti, R. M., and Radi, R. (1994) *Arch. Biochem. Biophys.* **310,** 118–125
51. Sies, H., Sharov, V. S., Klotz, L.-O., and Briviba, K. (1997) *J. Biol. Chem.* **272,** 27812–28817