

Differential Inhibitory Action of Nitric Oxide and Peroxynitrite on Mitochondrial Electron Transport

Adriana Cassina and Rafael Radi¹

Department of Biochemistry, Facultad de Medicina, Universidad de la República, 11800 Montevideo, Uruguay

Received October 12, 1995, and in revised form January 8, 1996

Various authors have suggested that nitric oxide ($\cdot\text{NO}$) exerts cytotoxic effects through the inhibition of cellular respiration. Indeed, in intact cells $\cdot\text{NO}$ inhibits glutamate-malate (complex I) as well as succinate (complex II)-supported mitochondrial electron transport, without affecting TMPD/ascorbate (complex IV)-dependent respiration. However, experiments in our lab using isolated rat heart mitochondria indicated that authentic $\cdot\text{NO}$ inhibited electron transport mostly by reversible binding to the terminal oxidase, cytochrome a_3 , having a less significant effect on complex II- and no effect on complex I-electron transport components. The inhibitory action of $\cdot\text{NO}$ was more profound at lower oxygen tensions and resulted in a differential spectra similar to that observed in dithionite-treated mitochondria. On the other hand, continuous fluxes of $\cdot\text{NO}$ plus superoxide ($\text{O}_2^{\cdot-}$), which lead to formation of micromolar steady-state levels of peroxynitrite anion (ONOO^-), caused a strong inhibition of complex I- and complex II-dependent mitochondrial oxygen consumption and significantly inhibited the activities of succinate dehydrogenase and ATPase, without affecting complex IV-dependent respiration and cytochrome c oxidase activity. In conclusion, even though nitric oxide can directly cause a transient inhibition of electron transport, the inhibition pattern of mitochondrial respiration observed in the presence of peroxynitrite is the one that closely resembles that found secondary to $\cdot\text{NO}$ interactions with intact cells and strongly points to peroxynitrite as the ultimate reactive intermediate accounting for nitric oxide-dependent inactivation of electron transport components and ATPase in living cells and tissues. © 1996 Academic Press, Inc.

Key Words: nitric oxide; superoxide; peroxynitrite; free radicals; mitochondria; cytochrome oxidase; nitrosothiols.

¹ To whom correspondence should be addressed at Departamento de Bioquímica, Facultad de Medicina Avda. Gral. Flores 2125, 11800 Montevideo, Uruguay. Fax: (5982) 949563. E-mail: RRADI@BQ.RAD.EDU.UY.

Nitric oxide ($\cdot\text{NO}$, nitrogen monoxide),² an endogenously synthesized radical intermediate, plays an important physiologic role in different organ systems (1–3). In spite of the physiologic role of $\cdot\text{NO}$, it has become evident that it can mediate cytotoxic actions either during the normal defensive function of neutrophils and macrophages toward target cells (4–6) or by excess production in tissues undergoing diverse pathological situations (7–9).

One important mechanism by which nitric oxide exerts cytotoxicity is the inhibition of cellular respiration secondary to the inactivation of mitochondrial aconitase and components of the electron transport chain (10–12). In particular, in intact cells $\cdot\text{NO}$ mediates inhibition of glutamate-malate (complex I) and succinate (complex II)-supported mitochondrial electron transport, without affecting TMPD/ascorbate (complex IV)-dependent respiration (11, 12).

Mammalian mitochondria is known to be a target for the toxic action of strong oxidants such as hydroxyl radical (13). However, $\cdot\text{NO}$ itself is a weak oxidant and it interacts with a limited number of biomolecules, in particular those containing heme moieties (14, 15). Nevertheless, $\cdot\text{NO}$ produced in aerobic environments can turn to a potent oxidant, by its reaction with superoxide ($\text{O}_2^{\cdot-}$), which yields peroxynitrite anion (ONOO^-) (16–19).

Peroxynitrite³ is a biologically generated reactive and toxic species produced under oxidative stress conditions (20–22). *In vitro* experiments have shown that biologically relevant concentrations of authentic per-

² Abbreviations used: $\cdot\text{NO}$, nitric oxide; $\text{O}_2^{\cdot-}$, superoxide; ONOO^- , peroxynitrite anion; TMPD, *N,N,N,N*-tetramethyl-*p*-phenylenediamine; Mops, 3-(morpholino)propanesulfonic acid; SNAP, *S*-nitroso-*N*-acetylpenicillamine; BSA, bovine serum albumin; RCR, respiratory control ratio; GSNO, *S*-nitrosoglutathione.

³ The term peroxynitrite is used to indicate either peroxynitrite anion (ONOO^-) or peroxynitrous acid (ONOOH) throughout the manuscript. The IUPAC recommended nomenclature for peroxynitrite is oxoperoxynitrate (1-), for peroxynitrous acid it is hydrogen oxoperoxynitrate and for nitric oxide it is nitrogen monoxide.

oxynitrite inactivate mammalian heart mitochondrial electron transport components and ATPase (23), with an inactivation profile remarkably similar to the one observed during $\cdot\text{NO}$ exposure to intact cells (24). In addition, Hausladen and Fridovich (25) and Castro *et al.* (26) have recently shown that mitochondrial aconitase is readily inactivated by peroxynitrite but not significantly by $\cdot\text{NO}$.

These considerations bring forth the possibility that the toxic effects of nitric oxide toward mitochondria observed *in vivo* may ultimately rely on the formation of secondary, more potent, nitrogen-derived oxidants such as peroxynitrite.

In this work we have studied the direct interactions of $\cdot\text{NO}$ and $\cdot\text{NO}$ plus O_2^- on intact rat heart mitochondria as evaluated by substrate-supported oxygen consumption, enzyme activities of individual electron transport components and ATPase, and visible absorption spectrum of cytochromes, with the aim of better defining the molecular mechanisms by which $\cdot\text{NO}$ mediates inhibition of cell respiration.

MATERIALS AND METHODS

Chemicals. Fatty acid free bovine serum albumin (fraction V), horse heart cytochrome c (type III), rabbit muscle pyruvate kinase (type II), rabbit muscle lactate dehydrogenase (type I), glutamic acid, malic acid, succinic acid, rotenone, antimycin A, ascorbic acid, potassium cyanide, *N,N,N,N*-tetramethyl-*p*-phenylenediamine (TMPD), NADH, 2,6-dichlorophenolindophenol, 3-(morpholino)propanesulfonic acid (Mops), 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes), ethylene glycol bis-(*B*-aminoethyl ether)*N,N*-tetracetic acid (EGTA), bovine erythrocyte hemoglobin, hypoxanthine, glutathione (reduced form), and allopurinol were obtained from Sigma (St. Louis, MO).

Xanthine oxidase from bovine milk was from Calbiochem. Bovine CuZn-superoxide dismutase was a kind gift of Grunenthal (Germany), *S*-nitroso-*N*-acetylpenicillamine (SNAP) was from BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA), and Sephadex G-25 was from Pharmacia Biotech Inc. (Sweden). High purity argon and nitric oxide gas were obtained from AGA Gas Co. (Montevideo, Uruguay).

Mitochondrial preparation. Adult rats (150–220 g) were anesthetized and hearts removed and washed extensively with cold homogenization buffer until they were free of blood, minced in small fragments, and homogenized by passing the tissue through a small tissue grinder. Tissue fragments were completely disrupted using a Potter–Elvehjem homogenizer in a homogenization buffer containing 0.3 M sucrose, 5 mM Mops, 5 mM potassium phosphate, 1 mM EGTA, 0.1% BSA. The homogenate was centrifuged at 1500*g* to eliminate large pieces of tissue and mitochondria were isolated from the supernatant by centrifugation at 10,000*g*. Mitochondrial pellets were resuspended in a minimal volume of homogenization buffer to 25–35 mg protein/ml and kept under ice at 4°C until used. Respiratory control ratio (RCR) for complex II-dependent respiration, typically ranged between 3 and 5.

Preparation of nitric oxide, SNAP, and S-nitrosoglutathione. A stock nitric oxide solution was obtained by flushing nitric oxide gas for 10 min in a gas-sampling tube containing high purity deionized water, which had been previously deoxygenated by extensive bubbling with argon for 30 min. The nitric oxide concentration achieved in solution was 1.5 mM at 25°C, which is close to saturation levels. Nitric oxide concentration was determined by measuring the oxidation of oxyhemoglobin to methemoglobin according to (27) or by elec-

trochemical detection using a nitric oxide sensor (Iso-NO, World Precision Instruments, Inc., Sarasota, FL).

S-nitrosoglutathione (GSNO) was synthesized at 25°C by reacting equimolar (200 mM) concentrations of reduced glutathione with sodium nitrite in 100 mM HCl, obtaining a reaction yield >95% (28). Solutions of SNAP were prepared in 25 mM H_2SO_4 (29). Peroxynitrite was synthesized from sodium nitrite and hydrogen peroxide using a quenched-flow reactor, as previously (17, 18). Excess hydrogen peroxide was eliminated with manganese dioxide. Peroxynitrite concentration was assayed spectrophotometrically at 302 nm ($\epsilon = 1.67 \text{ mM}^{-1} \text{ cm}^{-1}$).

Oxygen consumption studies. Mitochondrial respiration was measured polarographically using a Cole–Palmer oximeter fitted with a water-jacketed Clark-type electrode (YSI Model 5300) in a 1.0-ml reaction vessel. Oxygen consumption studies were performed in homogenization buffer at 37°C, pH 7.4, at 0.2–0.5 mg/ml mitochondria. Glutamate/malate (2.5 mM), succinate (5 mM), or ascorbate (5 mM) plus TMPD (0.5 mM) were used to quantitate complex I, II, and IV-dependent respiration, respectively.

Mitochondrial exposure to reactive species. For mitochondrial exposure to nitric oxide, intact mitochondria (1 mg/ml) and frozen-thawed mitochondria were incubated under anaerobic conditions in rubber-capped tubes in 100 mM phosphate buffer, pH 7.4, and were exposed to either authentic nitric oxide (5–200 μM) or *S*-nitrosothiols (GSNO, SNAP). Aliquots of the mitochondrial suspensions were taken through the rubber septum with gas-tight Hamilton syringes and mitochondrial-dependent oxygen consumption and enzymatic activities were measured. Also, exposure to nitrite and nitrate (5–200 μM) were performed.

Mitochondrial suspensions (2 mg/ml) were also exposed to aerobic incubations with 150 μM hypoxanthine plus 5.5 munits/ml xanthine oxidase which generate O_2^- and hydrogen peroxide (H_2O_2) for 30 min in 100 mM phosphate buffer, pH 7.4. The rates of formation of uric acid ($\epsilon_{292} = 11 \text{ mM}^{-1} \text{ cm}^{-1}$) and O_2^- , followed as the superoxide dismutase-inhibitable reduction of cytochrome c^{3+} ($\epsilon_{550} = 21 \text{ mM}^{-1} \text{ cm}^{-1}$), were measured spectrophotometrically. The univalent flux percentage was 19%, representing a superoxide flux of 4.2 $\mu\text{M}/\text{min}$. Xanthine oxidase-dependent reactions were stopped by addition of 100 μM allopurinol and mitochondrial suspensions washed once at 12,000*g* for 10 min, before running oxygen consumption studies.

A $\cdot\text{NO}$ flux was introduced in the reaction mixtures by the decomposition of GSNO (5 mM) as determined by oxidation of oxy- to methemoglobin at 577 nm ($\epsilon = 11 \text{ mM}^{-1} \text{ cm}^{-1}$; Ref. 27).

Enzyme assays. Succinate dehydrogenase was measured spectrophotometrically in the presence of 20 μM DCPIP, 15 mM succinate, and 2 μM KCN. Reduction of DCPIP was followed at 600 nm ($\epsilon = 20.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) (30). NADH dehydrogenase activity was quantitated by the rate of NADH-dependent ferricyanide reduction at 420 nm ($\epsilon = 1 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) (31), using 200 μM NADH and 0.5 mM ferricyanide in the presence of 5 μM rotenone. Cytochrome c oxidase activity was determined as the rate of oxidation of 30 μM reduced cytochrome c at 550 nm ($\epsilon = 20.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) (31). Reduced cytochrome c was prepared by reduction of cytochrome c^{3+} with excess sodium dithionite, followed by Sephadex-G25 chromatography. ATPase activity was assayed by coupling the reaction of pyruvate kinase with lactate dehydrogenase and following NADH oxidation at 340 nm ($\epsilon = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) (32). The assay system for ATPase activity contained 2.5 mM ATP, 50 $\mu\text{g}/\text{ml}$ pyruvate kinase, 50 $\mu\text{g}/\text{ml}$ lactate dehydrogenase in 50 mM Hepes, pH 8.

All enzymic determinations were performed either in a Kontron Analytical Model SFM 25 or a Shimadzu UV 160 spectrophotometers, using 0.1–0.2 mg/ml mitochondrial protein. Protein concentrations were determined using the method of Bradford (33).

Visible absorption differential spectra of mitochondrial cytochrome. The differential spectrum of mitochondria (1 mg/ml) under different experimental conditions was determined in the 500 to 630 nm region in 0.3 mM sucrose, 5 mM Mops, 5 mM potassium phosphate, 1 mM EGTA, pH 7.4, using a Spectronic 3000 Array spectrophotometer in

anaerobic cuvettes. A sample of mitochondria from the same preparation was used at the same concentration as the optical reference.

All the experiments reported in this manuscript were repeated with reproducible results a minimum of three times.

RESULTS

Inhibition of Mitochondrial Respiration by Nitric Oxide and Peroxynitrite

Sequential bolus additions of authentic $\cdot\text{NO}$ (5–15 μM) to mitochondrial suspensions in respiration buffer and at oxygen tensions ranging from 220 μM (air-saturated buffer) down to 65 μM , resulted on inhibition of complex I- and II-dependent, state-4 and state-3 (ADP-stimulated) respiration, an effect that was more evident at lower oxygen concentration in the respiration buffer (Figs. 1A–1G). Indeed, at 65 μM oxygen, 5 μM $\cdot\text{NO}$ caused near 100% inhibition of the rate of complex I and II-oxygen uptake in state-4 and -3 (Figs. 1B, 1C, and 1E–1G, respectively).

Interestingly, the inhibitory action of $\cdot\text{NO}$ was overcome by gentle bubbling of mitochondrial suspension with air for 10 s, resulting on 100% recovery of complex I- and IV-dependent oxygen consumption (Figs. 1B, 1C, and 2) and near 50% of complex II-dependent respiration (Figs. 1E–1G). Even when mitochondria were preincubated at high $\cdot\text{NO}$ concentrations (200 μM) and then diluted 5-fold for oxygen consumption studies (final $\cdot\text{NO}$ concentration 40 μM) reoxygenation resulted in 100% recovery of TMPD/ascorbate-dependent respiration (Fig. 2).

The transient inhibition by $\cdot\text{NO}$ did not result in mitochondrial uncoupling. For instance, after $\cdot\text{NO}$ displacement from mitochondrial suspensions following reoxygenation, addition of ADP (180 μM), to succinate-supported respiration resulted on state-3 respiration oxygen consumption rates similar to control mitochondria (Fig. 1E).

On the other hand, a flux of peroxynitrite inhibited complex I- and II-dependent respiration by 20 and 80%, respectively, without affecting complex IV-dependent respiration (Table I). The inhibitory effects of peroxynitrite on mitochondrial respiration were maintained after mitochondrial washings. This is different to what is observed with $\cdot\text{NO}$, since after mitochondrial washings only a marginal inhibition of complex I- and IV-dependent respiration remained, with a partial (40%) inhibition at complex II, which still was significantly less than that caused by a equimolar flux of peroxynitrite (80%) (Table I). Neither nitrite nor nitrate (up to 200 μM), the end products of $\cdot\text{NO}$ and peroxynitrite decomposition had any significant effect on mitochondrial respiration (data not shown).

Spectral Changes Induced by Nitric Oxide and Peroxynitrite

The visible differential spectrum (reduced minus oxidized) of dithionite-treated rat heart mitochondrial

preparation showed the typical absorption bands in the 500 to 650 nm region, corresponding to the reduced forms of the mitochondrial cytochromes (c, b, and aa_3) in the presence of sodium dithionite (Fig. 3A). Similarly, anaerobic incubation of mitochondria with $\cdot\text{NO}$ (50 μM) resulted in the appearance of bands corresponding to cytochrome aa_3 and c (Fig. 3B). Further addition of succinate to the $\cdot\text{NO}$ -treated mitochondria also made the cytochrome b band evident (Fig. 3C).

When mitochondria were exposed to succinate plus antimycin A, only the band of cytochrome b was evidenced (Fig. 3D), but interestingly, further addition of $\cdot\text{NO}$ (under anaerobic conditions) resulted in the appearance of an additional band corresponding to cytochrome aa_3 (Fig. 3E). The mitochondrial differential spectrum was not modified by either aerobic or anaerobic exposure to up to 1 mM authentic peroxynitrite (data not shown).

Inactivation of Mitochondrial Enzymes by Nitric Oxide Plus Superoxide

A peroxynitrite flux was obtained from the generation of equimolar fluxes of 4.2 $\mu\text{M}/\text{min}$ $\cdot\text{NO}$ from nitrosogluthatione and O_2^- from xanthine oxidase and resulted in the formation of peroxynitrite, as quantitated by the rate of oxidation of cytochrome c^{+2} to cytochrome c^{+3} , as reported recently (34). Under our assay conditions cytochrome c^{+2} (30 μM) oxidation yields using authentic peroxynitrite (10 μM) were 50%. Thus, a rate of cytochrome c^{+2} oxidation of 2.0 $\mu\text{M}/\text{min}$ corresponded to a peroxynitrite flux of 4.0 $\mu\text{M}/\text{min}$ (Fig. 4). Peroxynitrite formation was stopped by addition of the xanthine oxidase inhibitor, allopurinol. Indeed, allopurinol addition to the reaction mixture inhibited the oxidation of cytochrome c^{+2} by 90%.

Then, exposure of mitochondrial suspensions to a flux of 4.0 $\mu\text{M}/\text{min}$ peroxynitrite for 20 min resulted on a decrease of succinate dehydrogenase and ATPase activities by 50 and 86%, respectively, without inhibition of NADH dehydrogenase (Table II). However, exposures to independent fluxes of either nitrosogluthatione-derived $\cdot\text{NO}$ or xanthine oxidase-derived O_2^- did not result in lower enzyme activities of NADH dehydrogenase, succinate dehydrogenase, and ATPase than in the control samples (Table II). Moreover, aerobic or anaerobic addition of authentic $\cdot\text{NO}$ (60 μM) to mitochondrial suspensions did not result in inactivation of these enzymes after 20-min incubations. On the other hand, cytochrome c oxidase activity was inhibited after 20-min incubation with GSNO (5 mM aerobically) and authentic $\cdot\text{NO}$ (10 μM anaerobically) by 70 and 40%, respectively. The inhibitory effect of $\cdot\text{NO}$ on cytochrome c oxidase activity was significantly overcome in the presence of xanthine oxidase (Table II).

Neither nitrite nor nitrate (up to 200 μM) had inhibitory effects on the studied enzyme activities (not

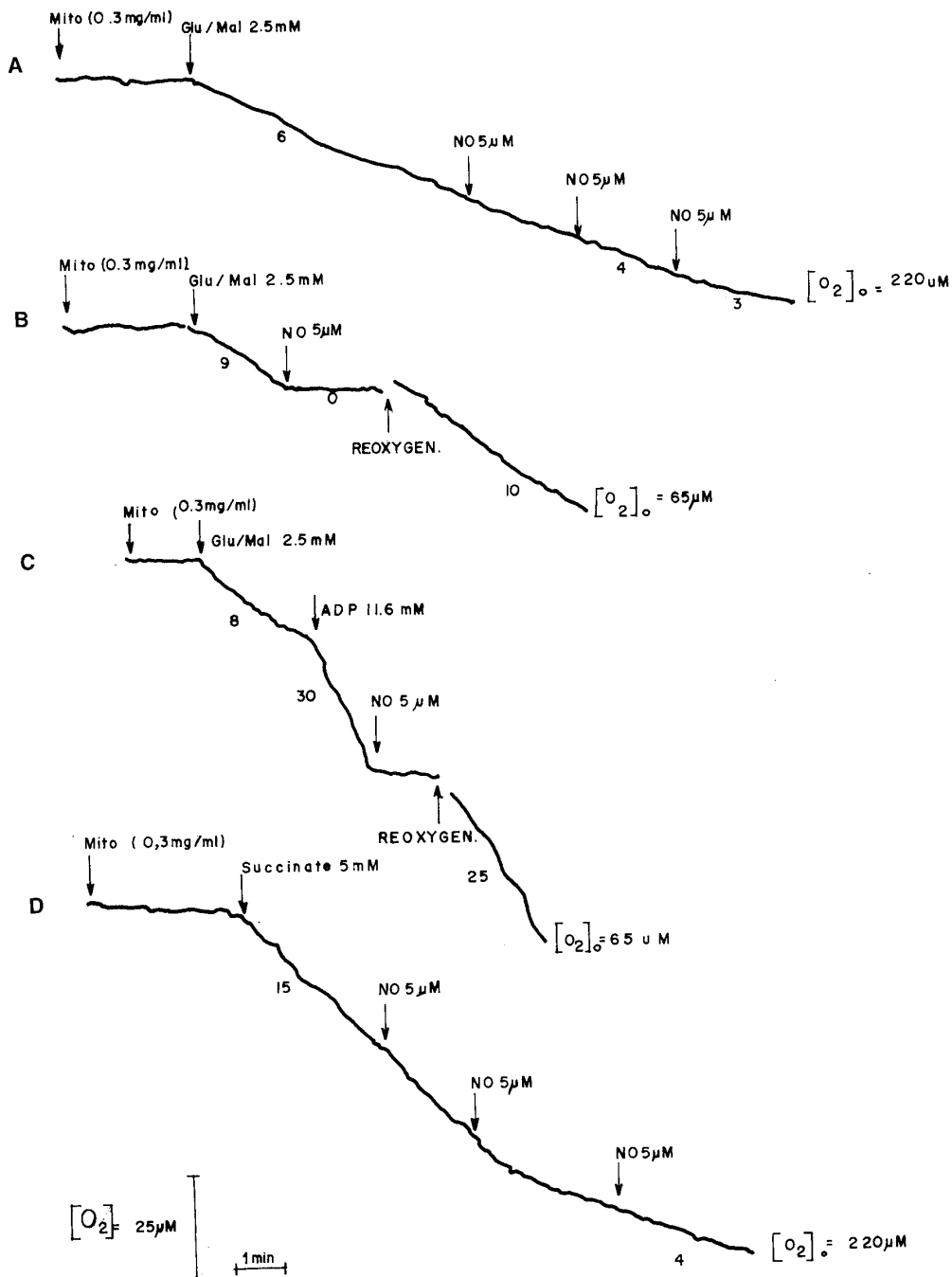


FIG. 1. Effect of $\cdot NO$ on substrate-dependent mitochondrial oxygen consumption. State-4 and state-3 oxygen consumption in presence of respiratory substrate and different concentrations of $\cdot NO$ was studied in mitochondria, at $220 \mu M$ (A,D) and $65 \mu M$ (B,C,E,F,G) initial oxygen concentration, using a polarographic Clark-type electrode at $37^\circ C$. The reaction vessel contained 1.0 ml final reaction volume. Concentration of added compounds are indicated in the figure. Numbers are rates of oxygen consumption in $nmol \cdot min^{-1}$. Note: Reoxygenation (REOXYGEN.) of the reaction mixture was accomplished by gentle bubbling for 10 s with air. Afterward, rate recording was restarted once oxygen concentration approached the same value existing right before reoxygenation.

shown) which correlates with the lack of effect on oxygen consumption.

DISCUSSION

In this work we have demonstrated a differential action of $\cdot NO$ and peroxynitrite on the activity of elec-

tron transport chain components and rates of oxygen consumption on isolated mammalian mitochondria. Inhibition of mitochondrial respiration by $\cdot NO$ was reversible and the effect was more pronounced at lower oxygen tensions (Fig. 1). Nitric oxide interaction with mitochondria caused inhibition of complex I-, II-, and

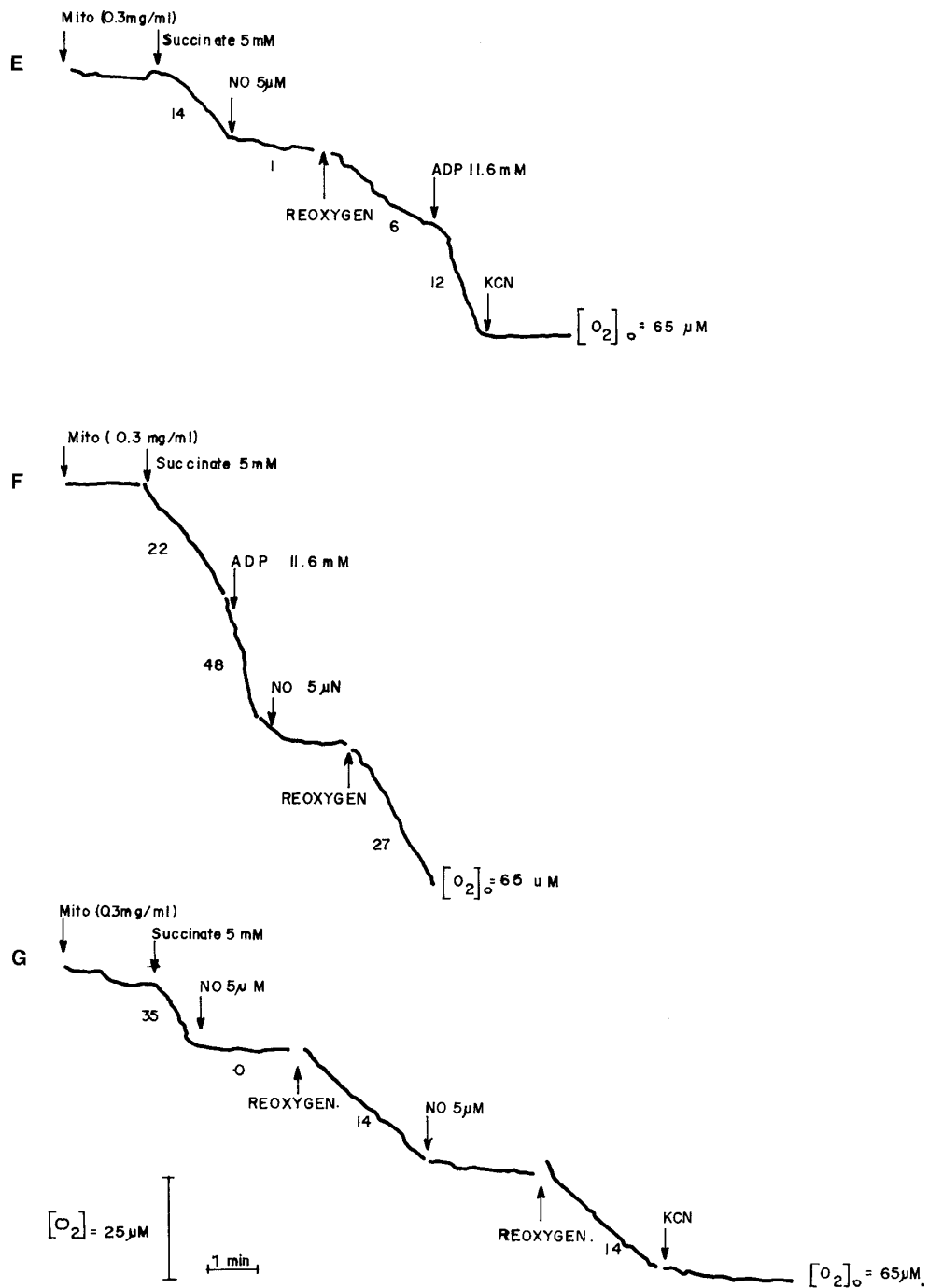


FIG. 1—Continued

IV-dependent respiration (Figs. 1 and 2). The inhibition could be overcome by removing \cdot NO from the mitochondrial suspensions through bubbling with air which returned oxygen consumption rates of state-4 and state-3 respiration to values similar to initial ones. The reversibility of \cdot NO inhibition was further evidenced by preincubation with high \cdot NO concentrations (200 μ M), since dilution and reoxygenation of mitochondrial sus-

pensions resulted on or near 100% recovery of TMPD/ascorbate (complex IV)-dependent oxygen consumption rates. In addition, the data indicate that \cdot NO did not cause significant uncoupling of mitochondria.

Considering the high affinity of \cdot NO for heme proteins, its ability to inhibit complex IV-dependent respiration (Fig. 2) and cytochrome c oxidase activity (Table I), and the competition observed with molecular oxy-

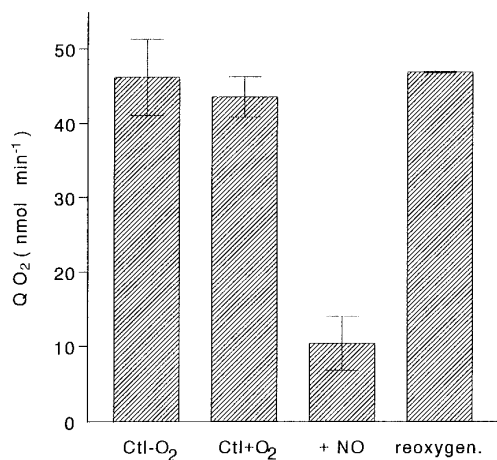


FIG. 2. Inhibition of TMPD/ascorbate-dependent mitochondrial respiration by nitric oxide. Mitochondria (1 mg/ml) were preincubated for 20 min at 25°C under the following conditions: aerobically (Ctl + O₂), anaerobically (Ctl - O₂), and anaerobically in the presence of 200 μM *NO (+*NO) and anaerobic preincubation in the presence of 200 μM *NO, which was then reoxygenated. Then, an aliquot of the mitochondrial suspension was taken, and complex IV-dependent oxygen consumption was studied at an initial O₂ concentration of 220 μM and 0.2 mg/ml mitochondrial protein concentration.

gen, we conclude that *NO binding to the heme moiety of cytochrome a₃ is one key mechanism accounting for *NO-mediated inhibition of mitochondrial respiration. This idea is in line with recent publications that show similar effects of nitrosothiols on brain mitochondria

TABLE I

Effect of Nitric Oxide and Peroxynitrite on Complex I, II, and IV-Dependent Oxygen Consumption after Mitochondrial Washings

Condition	% QO ₂		
	Complex I	Complex II	Complex IV
Control	100 ± 2	100 ± 3	100 ± 2
+ XO/HX	97 ± 2	93 ± 3	90 ± 2
+ GSNO	(30 ± 2) ^a	(15 ± 2) ^a	(17 ± 2) ^a
	93 ± 2	60 ± 2	90 ± 2
+ GSNO + XO/HX	80 ± 1	21 ± 1	97 ± 3

Note. Freshly isolated mitochondria (1 mg/ml) was incubated with xanthine oxidase (5 mU/ml) plus hypoxanthine (150 μM), GSNO (5 mM, rate of NO release = 5 μM/min) and xanthine oxidase plus hypoxanthine in the presence of GSNO in 100 mM potassium phosphate pH 7.4 for 30 min at 25°C. The reactions were inhibited by allopurinol (100 μM) and suspensions were washed once before running oxygen consumption studies in the presence of either, glutamate/malate, succinate, or TMPD/ascorbate. Control rates were 21, 103, and 115 nmol/min/mg for complex I, II, and IV, respectively. Data represent X ± SD, n = 3.

^a Data in parenthesis refer to studies performed prior to washing (thus GSNO present).

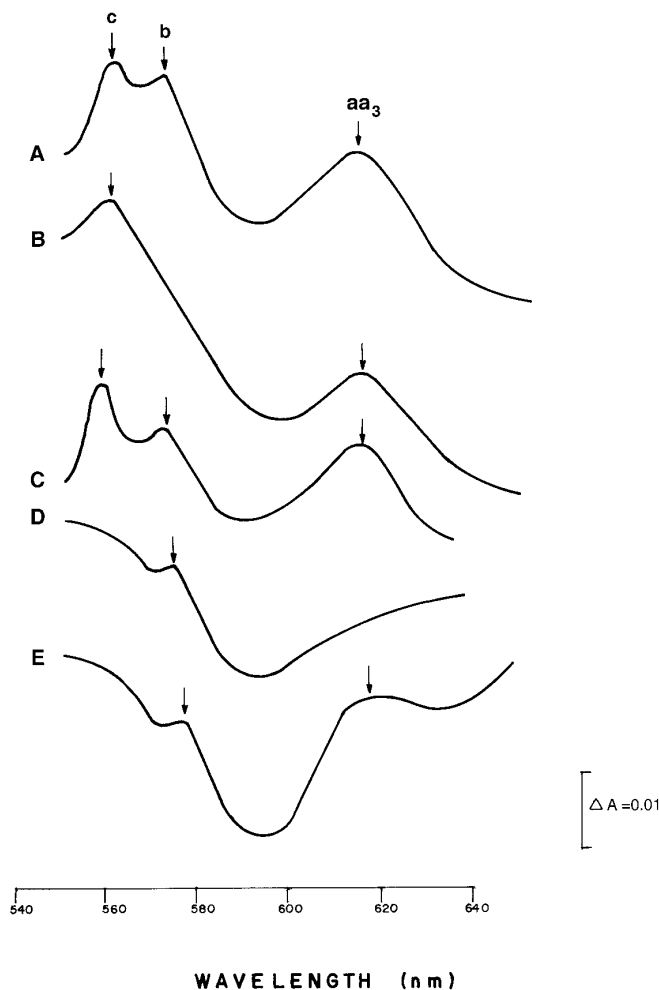


FIG. 3. Spectral changes in mitochondrial cytochromes induced by *NO. Traces represent mitochondria (2 mg/ml) with the following additions: (A) + sodium dithionite, (B) + *NO (50 μM) added anaerobically, (C) same as B plus succinate (2 mM), (D) + antimycin (2 μM) followed by sodium succinate (2 mM), and (E) + antimycin and succinate plus *NO (50 μM) added under anaerobic conditions.

(35) or authentic *NO in synaptosomal respiration (36). To further support this concept, our data show that *NO plus succinate resulted in a differential spectra similar to that observed in dithionite-treated mitochondria, in which cytochrome aa₃, b, and c were evidenced (Fig. 3). This was presumably due to the combination of two events, that is *NO binding to cytochrome a₃ and reduction of electron transport chain components by endogenous (Fig. 3B) or exogenously added substrate (Fig. 3C). Moreover, in addition to binding to a reduced form of cytochrome a₃, *NO may perform a one-electron donation to ferric cytochrome a₃ to form a nitrosyl-heme complex in which *NO has a character of nitrosonium ion (NO⁺) (37). Indeed, in succinate plus antimycin A-treated mitochondria, where only the cytochrome b is observed (Fig. 3D) and electron flow inhibited (38), addition of *NO resulted in the appearance

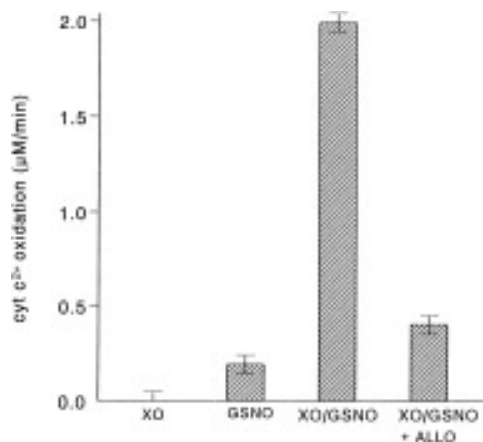


FIG. 4. Peroxynitrite formation from $\cdot\text{NO}$ plus O_2^- . Xanthine oxidase (5 mU/ml) plus hypoxanthine (150 μM) was incubated in the presence of GSNO (5 mM) in 100 mM potassium phosphate, pH 7.4, at 25°C. Peroxynitrite formation was detected by the rate of oxidation of cytochrome c^{+2} . Reaction was stopped by adding allopurinol (100 μM). Cytochrome c^{+2} oxidation by XO/GSNO corresponded to 4.0 $\mu\text{M}/\text{min}$ peroxynitrite (50% oxidation yield).

of the cytochrome aa_3 band (Fig. 3E), suggesting that $\cdot\text{NO}$ can directly reduce cytochrome a_3 under anaerobic conditions. Since $\cdot\text{NO}$ did not inactivate components of the electron transport chain (Table II), we conclude that the effect of $\cdot\text{NO}$ on mitochondrial respiration was mostly due to binding and inhibition at the cytochrome a_3 level, although some extent of permanent inhibition appears to be present in complex II also (Figs. 1E–1G and Table I).

On the other hand, it was previously shown that authentic peroxynitrite causes inhibition at complex I- and II- but not complex IV-dependent respiration and inactivates electron transport chain components, preferentially succinate dehydrogenase, and ATPase (23). In this work, mitochondria exposed to fluxes of O_2^- and

$\cdot\text{NO}$, which lead to peroxynitrite formation, had a pattern of inhibition of oxygen consumption (Table I) and enzyme inactivation (Table II) identical to the one previously obtained with pure peroxynitrite (23). This pattern shows a higher sensitivity of complex II in comparison to complex I to peroxynitrite (Table I). It was important to compare the outcome of mitochondrial electron transport exposed to a flux of O_2^- and $\cdot\text{NO}$ in comparison to pure peroxynitrite as we have recently observed that sometimes $\cdot\text{NO}$ may down-regulate O_2^- and peroxynitrite-dependent oxidative damage by participating in termination reactions with secondary radical intermediates (39). Thus, a rather striking conclusion is that inhibition of isolated mitochondrial respiration induced by peroxynitrite (and not by $\cdot\text{NO}$) is the one that closely resembles the inhibition pattern observed during $\cdot\text{NO}$ exposure to intact cells (24). The differential inhibitory action of $\cdot\text{NO}$ and peroxynitrite on the electron transport chain relies on molecular mechanisms comparable to those observed during their interaction with mitochondrial aconitase, in which $\cdot\text{NO}$ forms a transient inhibitory complex with an iron center of a protein (i.e., cytochrome a_3), while peroxynitrite causes oxidation and disruption of active sites (26).

Mitochondria is a key cellular source of O_2^- and we have shown before that peroxynitrite-mediated oxidative damage to mammalian mitochondria results in increased substrate-supported O_2^- formation (23). Nitric oxide reaction with mitochondrial-derived O_2^- would result in "site specific" formation and reactivity of peroxynitrite at the O_2^- generating sites, complexes I and II (38, 40). Thus, we postulate that $\cdot\text{NO}$ can initially bind to cytochrome a_3 producing a transient inhibition of normal electron flow (and ATP synthesis, Ref. 41) and promoting electron leakage to oxygen yielding O_2^- . Superoxide could then react with other $\cdot\text{NO}$ molecules diffusing to mitochondria to form peroxynitrite which would, in turn, cause inhibition of electron flow

TABLE II
Effect of Nitric Oxide and Peroxynitrite on Enzymic Activities

Condition	% Initial activity			
	NADH dehydrogenase	Succinate dehydrogenase	ATPase	Cytochrome c oxidase
Control	100 ± 5	100 ± 5	100 ± 9	100 ± 6
+ NO	88 ± 2	88 ± 3	90 ± 4	60 ± 9
+ GSNO	94 ± 3	95 ± 6	120 ± 10	32 ± 3
+ HX/XO	121 ± 10	116 ± 6	122 ± 14	105 ± 4
+ GSNO + HX/XO	102 ± 6	51 ± 6	12 ± 6	80 ± 3

Note. Mitochondria (1 mg/ml) was incubated with anaerobic NO (10 μM), GSNO (5 mM, rate of $\cdot\text{NO}$ release = 5 $\mu\text{M}/\text{min}$), and xanthine oxidase (5 mU/ml) plus hypoxanthine (150 μM) in 100 mM potassium phosphate, pH 7.4, for 30 min at 25°C. The reaction was inhibited by allopurinol (100 μM) and NADH dehydrogenase, succinate dehydrogenase, ATPase, and cytochrome c oxidase activity assayed spectrophotometrically. 100% activity corresponded to 400, 47, 99, and 41 $\mu\text{M}/\text{min}/\text{mg}$ for NADH dehydrogenase, succinate dehydrogenase, ATPase, and cytochrome c oxidase, respectively. Data represent $X \pm \text{SD}$, $n = 3$.

and enhanced $O_2^{\cdot-}$ formation, continuing a self-propagated peroxytrite-dependent injury process. In conclusion, our data support the concept that peroxytrite, and not $\cdot NO$, is responsible for the electron transport inhibition observed during $\cdot NO$ challenge to target cells and tissues.

ACKNOWLEDGMENTS

This work was supported by grants from CONICYT (Uruguay) and SAREC (Sweden). R.R. is an Established Investigator and A.C. is a M.S. student from PEDECIBA (Uruguay).

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