Nitric oxide inhibits mitochondrial NADH:ubiquinone reductase activity through peroxynitrite formation

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This study was aimed at assessing the effects of long-term exposure to NO of respiratory activities in mitochondria from different tissues (with different ubiquinol contents), under conditions that either promote or prevent the formation of peroxynitrite. Mitochondria and submitochondrial particles isolated from rat heart, liver and brain were exposed either to a steady-state concentration or to a bolus addition of NO. NO induced the mitochondrial production of superoxide anions, hydrogen peroxide and peroxynitrite, the latter shown by nitration of mitochondrial proteins. Long-term incubation of mitochondrial membranes with NO resulted in a persistent inhibition of NADH:cytochrome *c* reductase activity, interpreted as inhibition of NADH:ubiquinone reductase (Complex I) activity, whereas succinate:cytochrome *c* reductase activity, including Complex II and Complex III electron transfer, remained unaffected. This selective effect of NO and derived species was partially prevented by superoxide dismutase and uric acid. In addition, peroxynitrite mimicked the effect of NO, including tyrosine nitration of some Complex I proteins. These results seem to indicate that the inhibition of NADH:ubiquinone reductase (Complex I) activity depends on the NO-induced generation of superoxide radical and peroxynitrite and that Complex I is selectively sensitive to peroxynitrite. Inhibition of Complex I activity by peroxynitrite may have critical implications for energy supply in tissues such as the brain, whose mitochondrial function depends largely on the channelling of reducing equivalents through Complex I.

Key words: Complex I, hydrogen peroxide, superoxide anion, ubiquinol.

INTRODUCTION

Two membrane-bound flavoprotein dehydrogenases, NADH dehydrogenase and succinate dehydrogenase, channel reducing equivalents from the mitochondrial matrix to reaction centres in the mitochondrial respiratory chain. NADH dehydrogenase, a complex of at least 42 polypeptides ($\approx$ 900 kDa), constitutes Complex I and succinate dehydrogenase (120 kDa) constitutes Complex II [1]. NADH dehydrogenase was found to be more sensitive than succinate dehydrogenase when exposed to membrane-modifying agents, such as steroids and lysophospholipids [2–4]. Complex I was also more sensitive than Complex II in cancer cells that were exposed to macrophages; isolated mitochondria exhibited specific damage in NADH-dehydrogenase-dependent O_2 uptake due to the cytotoxic molecules, later recognized as peroxynitrite anion ($ONOO^-$), released by the macrophages [5,6].

NO, synthesized by different NO synthase isoforms, modulates mitochondrial respiratory functions by eliciting changes in O_2 consumption, energy-conservation processes and free-radical production [7]. The main regulatory action of NO in mitochondria relies on its reversible binding to cytochrome oxidase, which results in transient inactivation [8–10]. However, no other components of the respiratory chain have been reported to be transiently affected in experiments exposing mitochondria to micromolar pulses of NO. Conversely, it was recently observed that exposure of cultured macrophages to micromolar concentrations of NO for long periods (a condition that resembles inducible NO synthase activation in pathological situations), persistently inhibited respiration, probably through impairment of mitochondrial Complex I without affecting the activities of Complex II and III [11]. It was hypothesized that the deleterious effects of prolonged exposure to NO might derive from the formation of $ONOO^-$ in the vicinity of Complex I. In this context, the prolonged inhibition of cytochrome oxidase by NO promotes a highly reduced state of the upstream components of the mitochondrial electron-transfer chain, thereby facilitating the one-electron oxidation of ubiquinol by NO (eqn 1; $k_1 \approx 10^3\ M^{-1}\cdot s^{-1}$) [12]. Autoxidation of the semiquinone product in eqn (1) is an effective source of superoxide anion ($O_2^{\cdot-}$) in mitochondria (eqn 2):

$$UQH^- + NO \rightarrow UQ^{\cdot-} + NO^- + H^+ \quad (1)$$

$$UQ^{\cdot-} + O_2 \rightarrow UQ + O_2^{\cdot-} \quad (2)$$

where UQH^- is ubiquinol, $UQ^{\cdot-}$ is ubisemiquinone and UQ is ubiquinone. Depending on the relative concentrations of Mn-superoxide dismutase and NO, the fates of $O_2^{\cdot-}$ can be understood in terms of H_2O_2 (eqn 3; $k_3 \approx 2\times10^9\ M^{-1}\cdot s^{-1}$) [13] and $ONOO^-$ (eqn 4; $k_4 = 1.9\times10^{10}\ M^{-1}\cdot s^{-1}$) [14] formation [15].

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (3)$$

$$O_2^{\cdot-} + NO \rightarrow ONOO^- \quad (4)$$

Therefore, the effect of NO-derived oxidants on mitochondrial proteins could be expected to depend on the membrane ubiquinol and matrical Mn-superoxide dismutase concentrations (involving the reaction network mentioned above).

The purpose of this study is to assess the effects of long-term exposure to NO on respiratory activities in mitochondria from

Abbreviations used: $ONOO^-$, peroxynitrite anion; $O_2^{\cdot-}$, superoxide anion; DETA-NONOate, (2,2-hydroxynitrosohydrazino)bis-ethanamine.

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different tissues (with different ubiquinol contents), under conditions that either promote or prevent the formation of $ONOO^-$. The results presented herein support the notion that inhibition of Complex I by NO is accomplished through the intramitochondrial generation of $ONOO^-$.

MATERIALS AND METHODS

Chemicals and biochemicals

DETA-NONOate [(2,2-hydroxynitrosohydrazino)bis-ethanamine] was purchased from Research Biochemicals International (Natick, MA, U.S.A.). Acrylamide solutions, nitrocellulose membranes and goat anti-rabbit IgG were from Bio-Rad (Hercules, CA, U.S.A.). Anti-nitrotyrosine antibody was a generous gift from Dr Alvaro Estévez (University of Alabama at Birmingham, AL, U.S.A.). NO solutions (1.8 mM) were obtained by bubbling NO gas (99.9 % purity; AGA GAS, Maumee, OH, U.S.A.) in He-purged water at room temperature; the solutions were stored for a week at 4 °C. $ONOO^-$ was synthesized as described previously [16]. All other reagents were of analytical grade.

Isolation of mitochondria and preparation of submitochondrial particles

Excised liver, heart or brain from adult Sprague–Dawley female rats (200–250 g) were placed in an ice-cold homogenization medium consisting of either 0.23 M mannitol/70 mM sucrose/10 mM Tris/HCl/1 mM EDTA (for heart and liver) or 0.32 M mannitol/10 mM Tris/HCl/1 mM EDTA (for brain) with 0.5 % BSA (pH 7.4) and processed as described previously [9]. Submitochondrial particles were prepared from frozen and thawed mitochondria (20 mg of protein/ml) by sonication [9] and stored at −70 °C until use.

Oxygen consumption

O_2 uptake was determined polarographically with a Clark-type electrode placed in a 3 ml chamber at 30 °C, in an air-saturated 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_4/1 mM EDTA (pH 7.4; respiration medium) and either 1 mg of mitochondrial protein/ml or 0.1–0.3 mg of submitochondrial particle protein/ml. O_2 uptake was determined with either 6 mM malate/glutamate or 6 mM succinate as respiration substrates for Complex I or Complex II, respectively, in the presence (state 3) or absence (state 4) of 0.2 mM ADP. O_2 consumption was expressed in ngat (ng atom) of O/min per mg of protein.

Determination of NO

NO was determined amperometrically with an ISO-NOP electrode (World Precision Instruments, Sarasota, FL, U.S.A.) in 7 ml of respiratory reaction medium (as described above) at 30 °C. The NO electrode was calibrated daily with $NaNO_2$ in acid medium (0.1 M H_2SO_4/0.1 M KI) to generate a known concentration of NO.

Mitochondrial H_2O_2 production

H_2O_2 production was monitored continuously in a Hitachi F-2000 spectrofluorimeter (Hitachi, Tokyo, Japan) with excitation and emission wavelengths at 315 and 425 nm, respectively [17]. The respiration medium was supplemented with 6 mM succinate, 12.5 units/ml horseradish peroxidase, 50 μM *p*-hydroxyphenylacetic acid and 0.1 mg of submitochondrial-particle protein/ml. H_2O_2 production was initiated by the addition of a 1 μM pulse of NO.

Mitochondrial enzymic activities

The activity of Complexes I (NADH:ubiquinone reductase) and II (succinate:ubiquinone reductase) of submitochondrial particles at 0.05 mg of protein/ml was determined spectrophotometrically following the reduction of ubiquinone-0 (UQ-0; 2,3-dimethoxy-6-methyl-1,4-benzoquinone) at 268 nm with a Hitachi U3000 spectrophotometer at 30 °C; 0.1 mM NADH or 6 mM succinate were used as electron donors to assess Complex I or Complex II activity, respectively. Reactions were carried out in the presence of 1 mM KCN and expressed as nmol of reduced ubiquinol/min per mg of protein ($\epsilon_{268} = 14\,500\ M^{-1}\cdot cm^{-1}$). Complex I activity was selectively inhibited by 1 μM rotenone. Alternatively, reduction of 50 μM cytochrome *c* at 550 nm ($\epsilon_{550} = 21\,100\ M^{-1}\cdot cm^{-1}$) or NADH oxidation ($\epsilon_{340} = 5500\ M^{-1}\cdot cm^{-1}$) under the above-described experimental conditions was used to examine electron transfer through Complexes I–III and Complexes II–III. In experiments involving $ONOO^-$, the reaction medium consisted of 50 mM potassium phosphate/80 mM KCl/4 mM $MgCl_2$/1 mM EDTA, pH 7.4, to avoid unspecific scavenging of $ONOO^-$ by constituents of the above-described respiration medium; samples were diluted with the latter for measurements.

Immunoblotting and nitrotyrosine detection

Submitochondrial-particle proteins (40 μg) were separated by electrophoresis on precast SDS/polyacrylamide gels (7.5 %) and transferred to a PVDF membrane. Membranes were washed with a Tris-buffered saline solution, pH 7.4, containing 0.1 % Tween 20, and blocked with 4 % casein. They were then incubated with a rabbit polyclonal anti-3-nitrotyrosine antibody at a 1:2000 dilution for 1 h, washed and incubated with a second goat anti-rabbit IgG antibody conjugated to alkaline phosphatase at a 1:3000 dilution for 1 h, followed by detection of immunoreactive proteins by a chemiluminescence method.

Data analysis

Values in Tables and Figures are mean values from three to five determinations. Where appropriate, means ± S.E.M. are shown, and one-way ANOVA and Dunnets' test are used.

RESULTS

NO exposure inhibits Complex I-dependent O_2 uptake in mitochondrial membranes

Incubation of respiring rat liver mitochondria with a continuous flow of NO, reaching a steady-state level of 1 μM, resulted in a rapid inhibition of O_2 uptake (Figure 1A). Removal of NO upon addition of oxymyoglobin partially restored O_2 consumption supported by Complex I electron donors (malate/glutamate; Figure 1A, trace a), whereas it fully restored Complex II-driven respiration (succinate as electron donor; Figure 1A, trace b).

Exposure of rat liver submitochondrial particles to a steady-state level of NO resulted in the irreversible inhibition of respiration supported by NADH, the Complex I substrate; further supplementation with the Complex II electron donor, succinate, restored O_2 uptake, showing that Complexes II–IV were unaffected (Figure 1B, trace a). Accordingly, NO did not affect the O_2 consumption linked to succinate oxidation (Figure 1B, trace b).

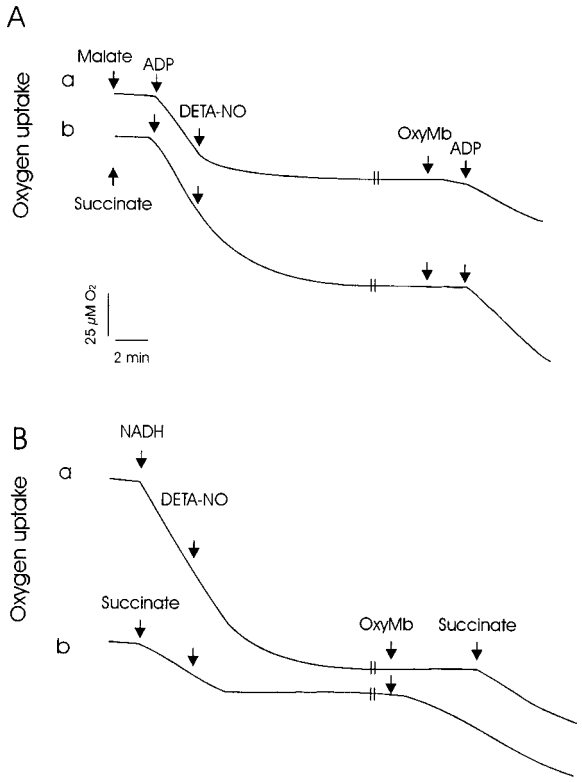

Figure 1 Effect of NO on the respiration of rat liver mitochondrial membranes

(**A**) Time course of O_2 uptake during the oxidation of Complex I or Complex II substrates by rat liver mitochondria. Assay conditions: rat liver mitochondria (1 mg/ml) suspended in respiration medium were supplemented with either 6 mM malate/glutamate (electron donors for Complex I; trace a) or 6 mM succinate (electron donor for Complex II; trace b) and 0.1 mM ADP. Then 1 mM DETA-NONOate was added when indicated by the arrows and incubated with the mitochondrial preparation for 20 min. NO was removed by further addition of 5 μM oxymyoglobin. The broken line (||) on the trace indicates the time necessary for DETA-NONOate to release NO until an equilibrium is reached ($\approx$ 8 min). (**B**) As in (**A**) but the assay was carried out with submitochondrial particles (0.1 mg of protein/ml) and NADH instead of mitochondria and malate/glutamate. Traces a and b correspond to particles supplemented with Complex I and II substrates, respectively. OxyMb, oxymyoglobin.

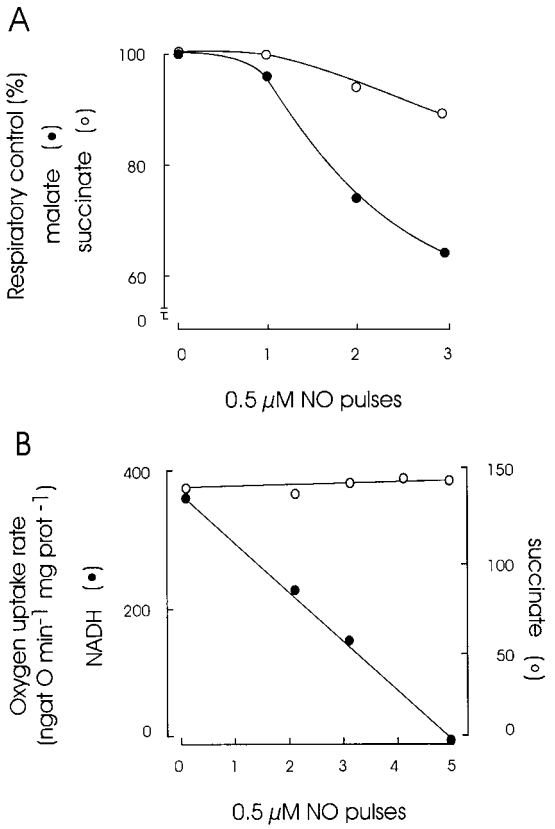

Figure 2 Effect of mitochondrial NO on respiratory control ratios and O_2 uptake

(**A**) Effect of NO on mitochondrial respiratory controls. Assay conditions: rat liver mitochondria (1 mg/ml) in respiration medium in the presence of either 6 mM malate/glutamate (●) or 6 mM succinate (○) were exposed to 1–5 pulses of 0.5 μM NO for 20 min periods. Respiratory controls were measured as described in the Materials and methods section. (**B**) Effect of NO pulses on O_2 uptake supported by Complex I (●) or Complex II (○) substrates. Assay conditions were as in (**A**) but with submitochondrial particles and NADH instead of mitochondria and malate/glutamate.

Addition of 0.5 μM NO pulses to either intact mitochondria or submitochondrial particles resulted in a progressive decrease of the respiratory control ratio, mainly through a decrease in state 3 (Figure 2A), and of the rate of O_2 uptake (Figure 2B) associated with pyridine-linked substrates; conversely, these parameters were not affected in mitochondria or submitochondrial particles respiring in the presence of succinate (Figure 2).

These findings, also observed in heart and brain mitochondria (results not shown), are consistent with a selective inhibition of mitochondrial Complex I activity by NO or derived products. The effects of NO on submitochondrial particles were prevented by the previous incubation with GSH; however, the thiol did not restore Complex I-driven respiration as previously reported on intact cells [18]: the inhibitory effect persisted at least up to 2 h after the removal of NO ($97 \pm 3\%$ inhibition of O_2 uptake in DETA-NONOate-treated submitochondrial particles versus $93 \pm 7\%$ inhibition after supplementation with 5 mM GSH).

NO-mediated formation of $O_2^{\cdot-}$ and $ONOO^-$ by mitochondrial membranes

The following studies establish a link between the steady-state levels of NO on the one hand and ubiquinol content, superoxide dismutase activity and nitrotyrosine accumulation on the other.

NO steady-state levels and ubiquinol content

We have previously reported [12] that the reaction of NO with mitochondrial ubiquinol led to $O_2^{\cdot-}$ formation, a process accomplished by NO-mediated ubiquinol oxidation (eqn 1), followed by ubisemiquinone autoxidation (eqn 2). Ubiquinol content in brain, liver and heart mitochondria may be estimated as 0.4, 1.0 and 1.5 mM, respectively, values obtained considering a mitochondrial volume of 3.6 μl/mg [19]. Hence, $O_2^{\cdot-}$ formation by mitochondria from these tissues is expected to differ as a consequence of their ubiquinol content: accordingly, the steady-state level of NO was displaced by mitochondria to lower values, the magnitude of the displacement being a function of the mitochondria ubiquinol content (Figure 3A). The steady-state level of NO reflects, on the one hand, the continuous formation of NO by the donor (DETA-NONOate) and, on the other, its consumption by mitochondrial components, mainly ubiquinol. This view is strengthened by the different H_2O_2 production by mitochondria from these tissues, also related to ubiquinol content (Figure 3A, insert). Mitochondrial H_2O_2 is expected to originate

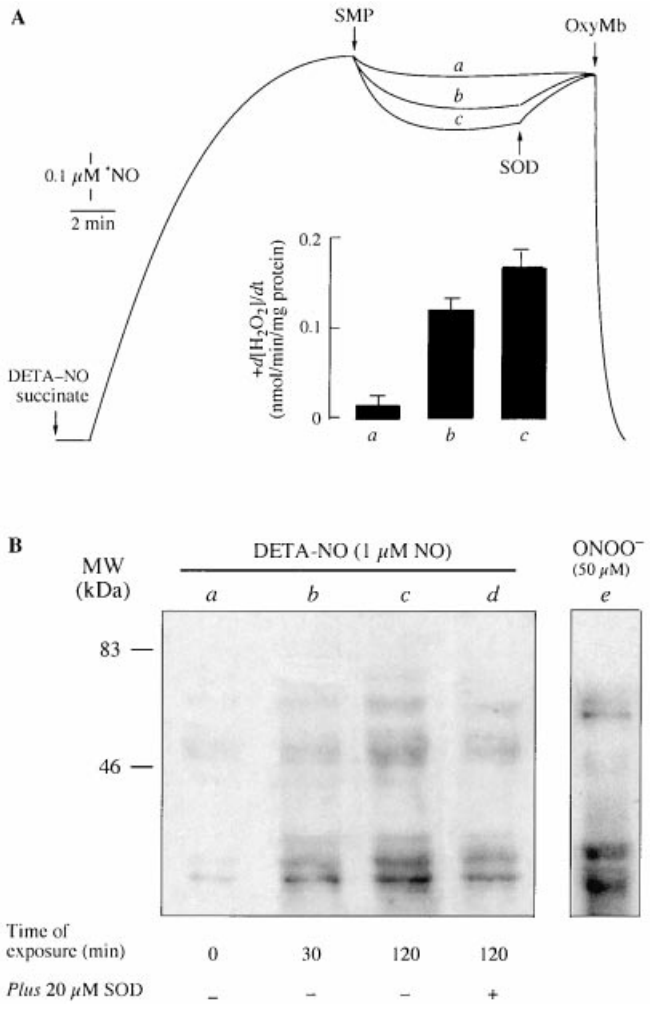

Figure 3 Effect of mitochondrial membranes on NO steady-state levels

(**A**) Effect of submitochondrial particles (SMP; 0.1 mg of protein/ml) from brain (trace a), liver (trace b) and heart (trace c) on the steady-state level of NO (1 μM) obtained with the NO donor DETA-NONOate. Reaction medium contained 6 mM succinate. Where indicated by the upward arrow, 3 μM superoxide dismutase (SOD) was added. The right-hand downward arrow indicates addition of 5 μM oxymyoglobin (OxyMb). Insert: mitochondrial production of H_2O_2 induced by NO. Submitochondrial particles, as in (**A**) above, were supplemented with 6 mM succinate and a 1 μM pulse of NO. Bars *a*, *b* and *c* correspond to submitochondrial particles from brain, liver and heart, respectively. (**B**) 3-Nitrotyrosine formation in rat liver submitochondrial particles exposed to steady-state NO for different periods. Assay conditions: submitochondrial particles (2 mg of protein/ml) in reaction medium were supplemented with 6 mM succinate (lane *a*) and subsequently exposed for 30 or 120 min to a steady-state concentration of NO of 1 μM (lanes *b* and *c*). Lane *d* represents the addition of 3 μM superoxide dismutase (SOD). Lane *e* shows the effect of a bolus addition of $ONOO^-$ on submitochondrial particles.

from the disproportionation of $O_2^{\cdot-}$ (eqn 3), which, in turn, is a product of ubisemiquinone autoxidation (eqn 2).

NO steady-state levels and superoxide dismutase

The steady-state level of NO was restored to the original level by superoxide dismutase (Figure 3A), thereby indicating that the reaction with $O_2^{\cdot-}$ (formed as in eqn 2 and leading to $ONOO^-$ formation; eqn 4) was the main decay pathway of NO. It may be surmised that two main pathways contribute to steady-state levels of NO in mitochondria: a reductive pathway involving

Table 1 Effect of NO on enzymic activities of submitochondrial particles

Assay conditions as described in the Materials and methods section. The concentration of submitochondrial particles was 0.5 mg of protein/ml. The steady-state concentration of NO was 1 μM. Incubation time was 20 min. SOD, superoxide dismutase.

	Enzyme activity (nmol of NAD^+/min per mg of protein)	
	NADH:cytochrome *c* reductase	Succinate:cytochrome *c* reductase
Control	85 ± 9	107 ± 10
Decayed DETA-NONOate	77 ± 2	–
DETA-NONOate (without succinate)	75 ± 5	105 ± 13
DETA-NONOate	19 ± 5*	113 ± 11
+ 20 μM SOD	33 ± 7*	–
+ 100 μM SOD†	76 ± 9	–
+ 20 μM SOD + 2 mM uric acid	47 ± 3*	–
+ 100 μM SOD + 2 mM uric acid†	78 ± 6	–
+ 5 mM GSH‡	80 ± 8	–
+ 5 mM GSH§	22 ± 9*	–

* $P < 0.05$ with respect to control values.

† Submitochondrial particles obtained in 100 μM Cu,Zn-SOD-supplemented sonication medium were used.

‡ GSH was included at the beginning of incubation with NO.

§ GSH was added after 20 min of incubation with NO and incubated for an additional 30 min.

oxidation of ubiquinol by NO (eqn 1) and an oxidative pathway involving its reaction with $O_2^{\cdot-}$ (eqn 4). These pathways are linked by ubisemiquinone autoxidation (eqn 2), thereby supporting a role of the mitochondrial ubiquinol pool in regulating NO levels.

NO steady-state levels and nitrotyrosine formation

Exposure of rat liver submitochondrial particles to a steady-state concentration of NO (1 μM) for 1–2 h in the presence of succinate resulted in the progressive nitration of tyrosine residues of mitochondrial proteins (Figure 3B, lanes *a–c*). This effect was prevented partially by superoxide dismutase (Figure 3B, lane *d*) or substrate deprivation. The extent of tyrosine nitration was comparable with that induced by a single bolus addition of 50 μM $ONOO^-$ (Figure 3B, lane *e*); some variations in the nitration pattern probably reflect the different reactivity of $ONOO^-$, either produced vectorially in mitochondrial membranes or added exogenously. These data are in agreement with the formation of $ONOO^-$ by mitochondrial membranes as a consequence of NO utilization.

Selective inhibition of Complex I by NO requires the production of $O_2^{\cdot-}$ and $ONOO^-$

The contribution of $ONOO^-$ to the inhibition of Complex I-driven respiration was examined in submitochondrial particles from brain and heart exposed to the same concentration of NO. As mentioned above, mitochondria from these tissues differ markedly in their $ONOO^-$ production in response to NO exposure.

Exposure of heart submitochondrial particles supplemented with succinate to a steady-state concentration of NO of 1 μM for 20 min resulted in a decrease of NADH:cytochrome *c* reductase activity of 75%, whereas succinate:cytochrome *c* reductase activity was not affected. No effects of NO were evident in the absence of an oxidizable substrate (Table 1).

The inhibition of NADH:cytochrome *c* reductase activity was partially prevented by the supplementation with either 20 μM

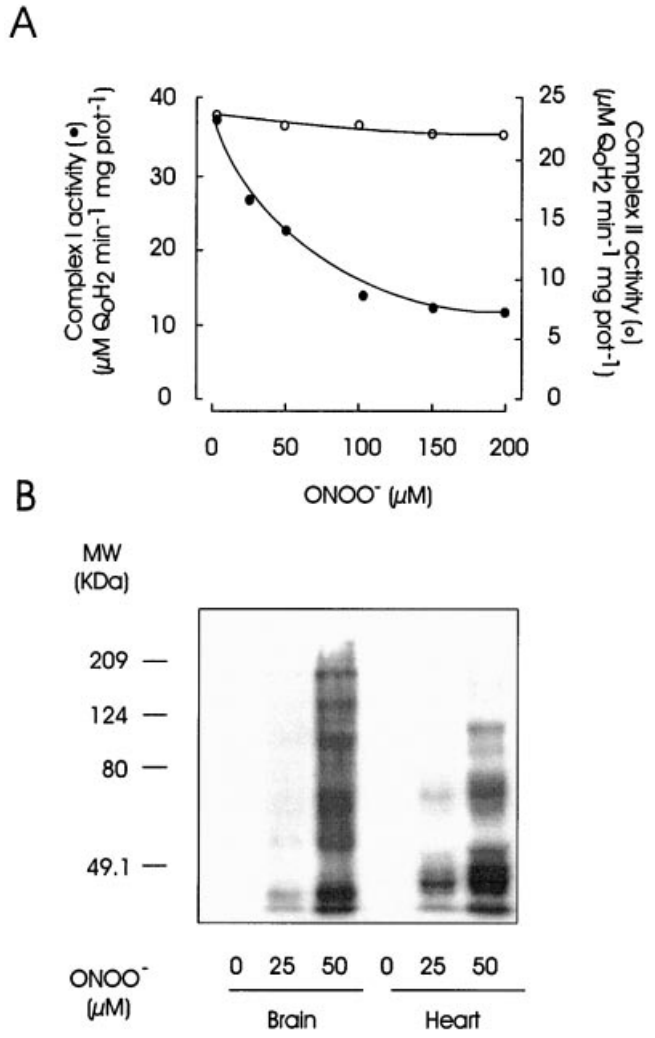

Figure 4 Effect of $ONOO^-$ on the activities of mitochondrial Complexes I and II

(**A**) Effect of increasing $ONOO^-$ concentrations on Complex I and Complex II activities. Assay conditions: brain submitochondrial particles (0.5 mg of protein/ml) in the reaction medium described in the Materials and methods section were supplemented with electron donors for Complex I and Complex II (0.1 mM NADH and 6 mM succinate respectively). (**B**) 3-Nitrotyrosine formation in brain and heart submitochondrial particles supplemented with a single dose of $ONOO^-$ (25 or 50 μM). Assay conditions were as in the Materials and methods section. Q_0H_2, reduced ubiquinol.

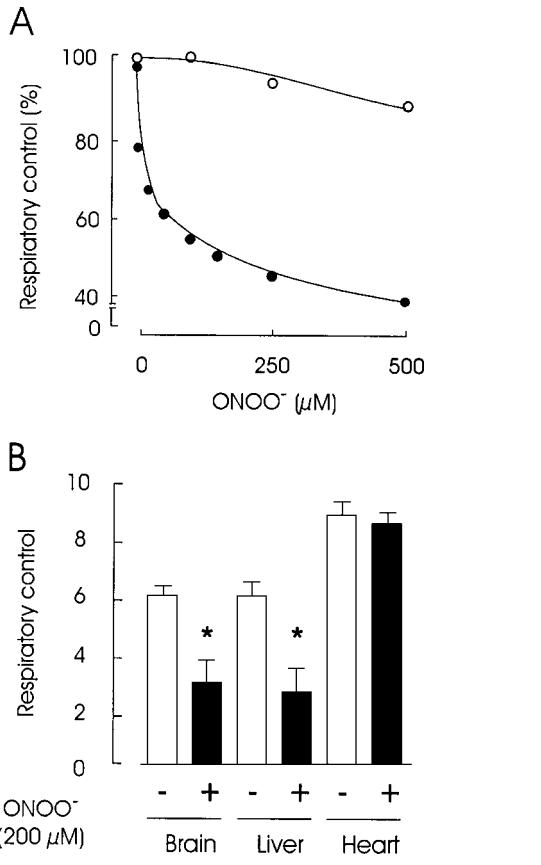

Figure 5 Effect of $ONOO^-$ on mitochondrial respiratory control ratio

(**A**) Effect of exogenous $ONOO^-$ on the respiratory control ratio of brain mitochondria in the presence of 6 mM malate/glutamate (●) or 6 mM succinate (○). Assay conditions were as in Figure 4(A). (**B**) Comparison of changes in respiratory control ratios of mitochondria in the presence of 6 mM malate/glutamate from different tissues elicited by 200 μM $ONOO^-$.

superoxide dismutase or 2 mM uric acid (a $ONOO^-$ scavenger). Addition of both superoxide dismutase and uric acid elicited a protective effect and restored as much as 61% of the control activity (Table 1). This partial effect of superoxide dismutase could be due to the inaccessibility of the enzyme to the space inside the submitochondrial particles in which $O_2^{\cdot-}$ is generated. Hence, submitochondrial particles were prepared with superoxide dismutase in the intravesicular space (by sonication of the mitochondria in a dismutase-containing medium). The assay was performed in the presence of 100 μM superoxide dismutase. Submitochondrial particles obtained as described above showed no inhibition of NADH:cytochrome *c* reductase activity upon incubation with DETA-NONOate (Table 1). Thus it may be surmised that the inhibitory effect of NO on NADH:cytochrome *c* reductase activity relies upon the production of mitochondrial $O_2^{\cdot-}$ and $ONOO^-$. The inhibition of NADH:cytochrome *c* reductase activity was not restored by further supplementation with 5 mM GSH, thereby suggesting that the effect of NO on isolated mitochondria did not involve S-nitrosylation. Neither diethyltriamine nor NO_2^-, which are released by 'decayed' DETA-NONOate, had a significant effect on the aforementioned enzyme activities.

Exposure of brain submitochondrial particles supplemented with succinate to the same steady-state level of NO resulted in similar, albeit of smaller magnitude, effects to those observed with heart submitochondrial particles (results not shown). To observe the same quantitative effect, rat brain submitochondrial particles required longer incubations with NO.

Exogenous $ONOO^-$ mimics the selective effects of NO on mitochondrial Complex I

Supplementation of brain submitochondrial particles with $ONOO^-$ resulted in a concentration-dependent inhibition of Complex I activity (NADH:ubiquinone reductase; Figure 4A). A half-maximal effect was obtained with 75 μM $ONOO^-$. A similar inhibition was observed with NADH:cytochrome *c* reductase activity (involving Complexes I and III). Complex II appeared to be less sensitive to the effects of $ONOO^-$, as revealed by the requirement of 550 μM $ONOO^-$ to elicit a half-maximal inhibition of succinate:ubiquinone reductase. Inhibition of succinate:cytochrome *c* reductase (Complexes II and III) activity

required similar high concentrations of $ONOO^-$ (Figure 4A). Brain and heart mitochondrial membranes also exhibited sensitivity to $ONOO^-$ (in terms of 3-nitrotyrosine formation) that was higher than that observed with heart (Figure 4B).

Exposure of intact brain mitochondria to increasing $ONOO^-$ concentrations resulted in a prompt decline of the respiratory control ratio when malate/glutamate was the electron donor; half-maximal inhibition was observed with 75 μM $ONOO^-$, a value similar to that required to inhibit NADH:ubiquinone reductase activity in submitochondrial particles. Conversely, with succinate as respiratory substrate (Figure 5A), no changes in the respiratory control ratio were observed. Figure 5(B) shows the relative effects of $ONOO^-$ on the respiratory control values of brain, liver and heart mitochondria. These effects of $ONOO^-$ accurately resembled those obtained in the presence of NO (described above) and are consistent with a selective and effective inhibition by $ONOO^-$ of Complex I, without substantial effects on Complexes II–IV.

DISCUSSION

These results furnished us with evidence for a selective inhibition of Complex I activity following NO utilization by mitochondria. The data were analysed within the context of the reactive species involved, the mechanisms inherent to Complex I damage and the differences afforded among mitochondria from different tissues.

The data in Figure 3 and Table 1 indicate that inhibition of Complex I activity is not due to NO itself but to species formed in tissue-specific mitochondrial pathways of NO metabolism. It is likely that $ONOO^-$ is the main species involved in damage of Complex I, a view supported by (i) the protective effects of superoxide dismutase and/or uric acid on Complex I activity (Table 1), thereby indicating the involvement of $O_2^{\cdot-}$ and $ONOO^-$, respectively, in the impairment of Complex I activity, (ii) the dose-dependent nitration of mitochondrial proteins observed upon exposure of mitochondrial membranes to NO and its attenuation by superoxide dismutase (Figure 3B) and (iii) the fact that the addition of $ONOO^-$ to submitochondrial particles resembled the effects of NO (Figure 4).

Although these results are consistent with an action of NO being mediated through $ONOO^-$ formation in the mitochondrial matrix, the mechanism underlying the selective inactivation of Complex I remains to be elucidated. In this study, the formation of nitrotyrosine suggests nitration of components of Complex I as the inactivating mechanism. However, the effects of $ONOO^-$ are rather complex and other mechanisms, such as S-nitrosylation of thiol groups, may contribute to Complex I inactivation. It has been reported previously [18] that in the presence of NO donors or $ONOO^-$, inhibition of Complex I activity in rat heart mitochondria is reversed by light or exposure to thiols. These observations led to the proposal that NO-derived $ONOO^-$ formation may reversibly nitrosylate thiol groups of Complex I components [18]. Moreover, these data are in line with previous observations in intact cells exposed to NO [11,20]. However, in the present studies, Complex I activity was not restored by addition of either GSH or dithiothreitol. Although GSH is hardly incorporated in intact mitochondria, reversion of inhibition by thiols was not detected in submitochondrial particles, which provide a convenient esteric disposition of the reactants. It is therefore likely that both $ONOO^-$-derived nitrosylation and nitration–oxidation of Complex I depend on the experimental conditions. In this context, the time of NO exposure could be critical. The oxidative-nitrative effects through $ONOO^-$ formation will be evident after most of GSH is oxidized by alternative pathways. Indeed, it was demonstrated that GSH content was a key factor determining the action of NO on Complex I [11,20]. In intact cells with an active biosynthetic machinery, GSH may be kept at a high-enough concentration to sustain transnitrosylation and the S-nitrosylation pathway. This effect should be reversed by thiol addition or photolysis. In isolated organelles, the endogenous GSH pool would favour a similar mechanism provided that the time of exposure to NO is sufficiently brief. This would also be the case if there is available substrate to sustain GSH homoeostasis. However, in our experimental model, the more prolonged time of NO exposure (doubling that of Borutaité et al. [18]), or addition of $ONOO^-$ without a substrate, appeared to favour the irreversible nitrative-oxidative mechanism. Moreover, a sustained production of H_2O_2 and derived oxidants is expected to contribute to deplete the GSH pool of the organelles via glutathione peroxidase. Accordingly, in Parkinson's disease, a decrease in GSH levels is associated with permanent Complex I impairment and tyrosine nitration in dopaminergic neurons.

The mitochondrial formation of $ONOO^-$ is a consequence of increased matrical steady-state levels of NO and $O_2^{\cdot-}$ [21]. The effects of NO on mitochondrial free-radical generation are complex; the NO-induced mitochondrial production of $O_2^{\cdot-}$, H_2O_2 and $ONOO^-$ and its further modulation are initiated by the redox reaction of NO and ubiquinol (eqn 1), leading to the formation and subsequent oxidation of ubisemiquinone (eqns 2–4) [12]. The contribution of eqn (1) to the mitochondrial formation of the aforementioned oxidants depends on the concentrations of NO and ubiquinol (ubiquinol as *pro-oxidant*) and on the rates of other reactions of NO utilization, like $ONOO^-$ formation. The role of ubiquinol as an antioxidant and anti-nitrating compound gains significance when its reaction with $ONOO^-$ is considered (eqn 5) [22]:

$$UQH^- + ONOO^- \rightarrow UQ^{\cdot-} + HO^- + NO_2 \quad (5)$$

As a result, the observed differences in NO-derived $O_2^{\cdot-}$ and H_2O_2 production rates among brain, liver and heart mitochondria (Figure 2) may be attributed to the different ubiquinol contents of these organelles [15]. However, the extent of $ONOO^-$-induced inhibition of Complex I activity in different mitochondria should reflect a balance upon activities of eqns (1)–(4) and (5), both depending on the ubiquinol concentration. In agreement with this notion, brain mitochondria, with the lowest ubiquinol content, required a prolonged exposure to NO to damage Complex I; in addition, a low ubiquinol content rendered the mitochondrial components more sensitive to the effects of exogenous $ONOO^-$. The relative participation and rates of eqns (1) and (5) will depend on NO concentration: at the highest NO concentration, most of the $O_2^{\cdot-}$ will be converted to $ONOO^-$ rather than to H_2O_2 (via superoxide dismutase) [15]. Consequently, most of the NO will be converted to $ONOO^-$. In this way, eqn (1) functions as a chemical reactor initiating the NO-derived oxidative machinery; at 1 μM NO, however, no more than 5–10 % of the rate of NO utilization should depend on eqn (1). This assumption is experimentally supported by the almost complete recovery of initial NO steady-state concentration by supplementing active mitochondrial particles with superoxide dismutase (Figure 3). Furthermore, ubisemiquinone autoxidation at Complex III [23] is not the sole source of $O_2^{\cdot-}$ in mitochondria; albeit at lower rates, autoxidation of Complex I flavins yields $O_2^{\cdot-}$ [24]. The selective inhibition of Complex I activity during NO metabolism may modify this additional mitochondrial source of oxidants.

This study also shows that Complex II activity is not affected by NO or $ONOO^-$ in either intact mitochondria or submitochondrial particles. This observation differs from a previous

study [25], which reported non-inhibitory effects of $ONOO^-$ on NADH:ferricyanide reductase activity. The discrepancy may be bridged by considering that, in that case, the experimental procedure could involve direct electron transfer from the flavin to ferricyanide, thereby bypassing the reductase domain of NADH dehydrogenase. The NADH:ubiquinone reductase activity measured in this study strongly suggests that $ONOO^-$ reacts with the ubiquinone reductase domain of Complex I. Moreover, inhibition was specifically referred to rotenone-sensitive electron flow through Complex I; measurement of activities other than those obtained in the presence of rotenone could inadvertently lead to include non-specific redox reactions in the experimental system.

Inhibition of Complex I activity was previously reported in studies with macrophages [11]. Intact cells require a longer exposure to NO than isolated mitochondria; half-maximal inhibitions of Complex I activity in macrophages and mitochondria are achieved at 180 and 15 min of NO exposure, respectively. This difference is probably attributable to a significant NO utilization by cytosolic factors in intact cells, which, in turn, restricts the NO availability to the mitochondrial matrix.

Inhibition of Complex I could be critical for those tissues, like brain, that strictly channel reducing equivalents (such as NADH) into site I. Hence, inhibition of Complex I may represent a stepwise process in the pathophysiology of different illnesses [11]; brain tissue exhibits an inhibition of mitochondrial Complex I in Parkinson's disease [26] and mitochondrial-permeability-transition-pore-induced parkinsonism [27]. Under these conditions, nitration of proteins, a fingerprint of $ONOO^-$ effects, has recently been found in the central nervous system [28]. Moreover, chronic administration of the Complex I inhibitor rotenone was shown to reproduce specific changes in the central nervous system ascribed to Parkinson's disease [29]. The understanding of long-term actions of NO on mitochondria will be important for the development of rational therapeutic approaches to neurodegenerative disorders.

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