Functional implications of nitric oxide produced by mitochondria in mitochondrial metabolism

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The effects of endogenous production of NO[•], catalysed by the mitochondrial nitric oxide synthase (NOS), on mitochondrial metabolism were studied. The respiratory rates of intact mitochondria in State 4 were decreased by 40% and 28% with succinate and malate–glutamate, respectively, in the presence of L-arginine (L-Arg); conversely, the O₂ uptake with N^G-methyl-L-arginine (NMMA), a competitive inhibitor of NOS, was increased. The production of NO[•] and the inhibition of the respiratory rates were dependent on the metabolic state in which mitochondria were maintained: NO[•] production was probably supported by mitochondrial NADPH, the latter maintained by

the energy-dependent transhydrogenase. In addition to the decline in the respiratory rate, an inhibition of ATP synthesis was also observed (40–50%) following supplementation with L-Arg. The dependence of the respiratory rates of mitochondria in State 3 and cytochrome oxidase activities on O₂ concentrations with either L-Arg or NMMA indicated that both processes were competitively inhibited by NO[•] at the cytochrome oxidase level. This inhibition can be explained by the interaction of NO[•] with cytochrome oxidase at the binuclear centre. The role of NO[•] as a physiological modulator of cytochrome oxidase is discussed in terms of cellular metabolism.

INTRODUCTION

Nitric oxide (NO[•]) is a free radical produced during the 5-electron oxidation of L-arginine (L-Arg) to citrulline catalysed by nitric oxide synthase (NOS) [1,2]. NO[•] has been reported to play an important role in neurotransmission, immune response and vasodilation in a variety of biological tissues [3–5].

The molecular target through which NO[•] exerts its effects on cells appears to be the soluble guanylate cyclase: binding of NO[•] to this enzyme triggers a series of events initiated by cGMP [6]. However, the inhibition of respiration by NO[•] in a variety of biological systems [7–11] has led us and others to suggest the involvement of an additional target in mitochondria, i.e. modulation of the respiratory rate by NO[•] might produce a series of events which may be considered to be as relevant as those mediated by guanylate cyclase activation. Nevertheless, conclusions regarding the role of NO[•] in mitochondria under *in vivo* conditions have been obtained indirectly because of the limitations of the experimental procedures used, i.e. in earlier studies, the biological systems were exposed to NO[•] from exogenous sources, namely from NO[•]-producing cells [11–13], NO[•] donors [10] or pure NO[•] gas [9,10,14,15]. In particular, this use of pure NO[•] gas prevented the effects of species released during the formation of NO[•] from either NO[•] donors (e.g. thiyl radical, disulphide anion radical, and O₂^{•-} derived from nitroso-glutathione; cyanide from nitroprusside) or stimulated immunocells (e.g. O₂^{•-}, H₂O₂, HOCl). However, the bolus addition of NO[•] may trigger a different set of biological effects than those expected from such a local, slow and constant production of NO[•]. All these conditions led to reports of a wide range of NO[•]-effective concentrations from nM to μM levels [16], with some falling close to cytotoxic levels, thereby leaving a small margin for the study of regulatory/modulatory processes. In addition,

various components of the mitochondrial respiratory chain (Complex I, iron–sulphur clusters, cytochrome oxidase; [9,13,17]) were identified as targets, and different mechanisms for NO[•] effects (competitive and non-competitive binding, irreversible damage; [9,13]) were proposed.

Recent findings of a mitochondrial (mt)NOS present at the inner mitochondrial membrane [18,19] circumvents earlier-noted limitations in the experimental models used, offering a unique possibility of studying directly the effects of an endogenous production of NO[•] on the parameters of mitochondrial function. In the present study, the respiratory rate, cytochrome oxidase activity and ATP synthesis were evaluated in intact coupled rat liver mitochondria under conditions of an endogenous flux of NO[•].

The results reported in this study support the idea that NO[•], produced by intact mitochondria, modulates the respiratory rate and ATP synthesis by inhibiting cytochrome oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1). NO[•] is catabolized by cytochrome oxidase to NO₂⁻ and NO₃⁻, and probably to other products not detected by the Griess assay. This catabolism at the cytochrome oxidase level is consistent with the transient depolarizations observed with isolated mitochondria exposed to pure NO[•] gas. The role of NO[•] as a physiological modulator of cytochrome oxidase, as well as that of peroxynitrite (ONOO⁻), is discussed relative to cellular metabolism.

MATERIALS AND METHODS

Materials

EDTA, EGTA, sodium succinate, sodium malate, sodium glutamate, mannitol, sucrose, CHAPS, Hepes, ATP, ADP, NADH, NADPH, toluene, NaNO₂, KNO₃, sulphanilamide, *N*-(1-

Abbreviations used: (mt)NOS, (mitochondrial) nitric oxide synthase; SMP, submitochondrial particle; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; NMMA, N^G-methyl-L-arginine; RCR, respiratory control rate; SOD, superoxide dismutase.

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naphthyl)ethylenediamine and BSA (fatty-acid free) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Catalase, superoxide dismutase and nitrate reductase (*Aspergillus* sp.) were obtained from Boehringer Mannheim (Mannheim, Germany). Oxymyoglobin was obtained as follows: 1 mM of commercially available horse heart myoglobin, dissolved in 0.1 M Hepes buffer, pH 7.4, was mixed with a 2 M excess of sodium dithionite to obtain deoxymyoglobin. Removal of remaining dithionite was achieved by gel chromatography [20].

Isolation of rat mitochondria

Rat liver mitochondria were isolated by differential centrifugation, purified by Percoll centrifugation [18,19] and washed twice with 150 mM KCl. Permeabilized mitochondria were obtained by a controlled treatment with toluene, as described previously [18]. Submitochondrial particles (SMPs) were obtained by sonication [18].

Biochemical analyses

Detection of NO[•] production

The oxidation of oxymyoglobin to metmyoglobin by NO[•] was monitored at 581–592 nm in a Hitachi U-3110 double-beam spectrophotometer with a multiple wavelength programme set at 22 °C. The rates of oxymyoglobin oxidation, sensitive to *N*^G-methyl-L-arginine (NMMA) inhibition and evaluated in the presence of catalase [18], were used as the rates of NO[•] production. The reaction medium used with intact mitochondria consisted of 0.225 M sucrose, 5 mM MgCl₂, 20 mM KCl, 10 mM potassium phosphate, 20 mM Hepes/KOH, pH 7.4 ('reaction medium') supplemented with 50 μM oxymyoglobin. The reaction was started with the biological material. The 'reaction medium' used with SMPs (0.4 mg protein/ml) and permeabilized mitochondria consisted of 'reaction medium' with the addition of 0.2 mM L-Arg, 0.5 mM NADPH, 4–5 μM tetrahydrobiopterin, the equivalent of 20–50 nM FMN and, where indicated, 10 mM succinate. The requirement for calmodulin varied with different SMP preparations.

Oxygen consumption

Oxygen uptake was measured polarographically with a Clark-type electrode (Yellow Springs Instruments, OH, U.S.A.) calibrated with air-saturated water at 35 °C. Sodium dithionite was added to obtain an experimental zero O₂ concentration. For different O₂ concentrations, the 'reaction medium' was deoxygenated under a stream of purified N₂. Injections of O₂-saturated buffer were administered successively to produce the desired final concentrations. The experimental calibration curve was linear in the range from 0.1 to 250 μM O₂ (*r* = 0.98), and 9 ± 2 mV corresponded to 1 μM O₂ at 2 V full scale. At low O₂ concentrations, the full-scale setting was changed to obtain an optimal response, and if the baseline drift was higher than 1 %/min, it was quantified and subtracted from the experimental rates. The sensitivity of the instrument was improved, and the response time (which usually is 15 s when switched from N₂ to air) was shortened by using a thinner membrane (Teflon membrane of 1.25 μm thickness; Rank Brothers, Cambridge, U.K.), and by replacing the membrane daily. In addition, the residual current was decreased by careful cleaning the electrode. The back-diffusion of O₂ to the chamber was decreased by sealing the Lucite plunger access slot and by wrapping Teflon tape around the screw part of the plunger. The stirring speed was set at approx. 500 rev./min, and to avoid electrical interference a small capacitor (5–10 μF) was put across the recorder terminals.

Cytochrome oxidase activity

Cytochrome oxidase activity was measured in permeabilized mitochondria by following the O₂ consumption in the presence of 0.2 μg/ml antimycin, 1.5 mM ascorbate and 0.3 mM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine in 'reaction medium' [21].

Measurement of ATP, ATP synthesis and consumption

Mitochondria (0.5–1 mg/ml) incubated in 'reaction medium' and supplemented with 10 mM succinate and 0.8 mM ADP were incubated in the O₂ chamber as described previously. Aliquots were withdrawn with a microsyringe at different time points to evaluate the amount of ATP synthesized. In separate experiments, the total amount of ATP synthesized was evaluated by taking aliquots at the end of State 3 (during the transition of State 3 → State 4). In both procedures, ATP was measured spectrophotometrically as described by Bücher [22] and modified by Adams [23]. The ATPase activity was measured using toluene-permeabilized mitochondria (0.2 mg/ml) incubated in 'reaction medium' supplemented with 8.5 % (v/v) poly(ethylene glycol) (molecular mass 8 kDa), 0.1 mM NADPH, 1 mM CaCl₂ in the absence (–L-Arg) and presence of 80 μM L-Arg (+L-Arg). Control experiments included incubations with only NADPH, CaCl₂ or L-Arg. After 5 min of incubation at 37 °C, 1 mM ATP was added to all the preparations, and the remaining ATP was measured as described above in aliquots taken every min for 10 min.

Nitrate and nitrite determination

SMPs (0.4 mg protein/ml) were incubated with 0.2 mM L-Arg, 0.5 mM NADPH, 4–5 μM tetrahydrobiopterin, the equivalent of 20–50 nM FMN and, where indicated, 10 mM succinate. Aliquots (0.5 ml), taken at different time points, were centrifuged at 12000 *g*, and the supernatants were incubated with 0.1 mM NADPH and 150 units/ml nitrate reductase for 30 min at 22 °C. The NO₂[–] formed was incubated for 10 min with 1 % (v/v) sulphanilamide in 30 % (v/v) acetic acid and 0.1 % (w/v) *N*-(1-naphthyl)ethylenediamine in 60 % (v/v) acetic acid to produce a diazo dye that absorbs at 540 nm. The amount of NO₃[–] was calculated from calibration curves constructed using standard solutions of NO₃[–] and NO₂[–].

NADPH evaluation

NADPH levels were evaluated by two different techniques: (i) the changes in pyrimidine nucleotide levels were evaluated by following the fluorescence of intact mitochondria at λ_{excitation} 340 nm and λ_{emission} 450 nm respectively [24]; although this method evaluates the levels of both NADH and NADPH, the level of the former is about 1/10 of that of NADPH [25,26]; (ii) the levels of NADPH were evaluated by HPLC analysis in KOH extracts of mitochondria, as described for brain tissue [27]. The correlation between the two methods was 0.90.

Protein determination

Protein was determined by the Lowry assay using BSA as a standard [28].

Data evaluation

All assays were done in duplicate and were repeated twice in separate experiments using 2 rats per experiment. Data are presented as means ± S.D.

RESULTS AND DISCUSSION

Effect of nitric oxide on oxygen consumption and oxidative phosphorylation in isolated mitochondria

Intact coupled mitochondria [respiratory control ratio (RCR) = 6.5 ± 0.5] were isolated by differential centrifugation from rat liver and purified by Percoll centrifugation. The latter procedure yielded mitochondria minimally contaminated with other sub-cellular compartments, and with higher RCRs than those obtained only by differential centrifugation [18]. These mitochondria were used as a source of NO[•] and to evaluate the effect of NO[•] on mitochondrial functional parameters.

The dependence of O₂ consumption on L-Arg concentration was measured in mitochondria oxidizing either succinate or malate–glutamate as substrates (Figure 1A). As the L-Arg concentration increased, there was a progressive decline in the respiratory rate: at 100 μ M L-Arg (0.1 μ mol/mg protein), the respiratory rate decreased by 40% and 28% oxidizing succinate and malate–glutamate respectively (Figure 1A). Higher concentrations of L-Arg (above 40–50 μ mol/mg of protein) were found to significantly decrease the RCR of intact mitochondria by increasing the rate of O₂ consumption in State 4. Inhibition of the respiratory rate by L-Arg supplementation was reversed by adding 50–100 μ M oxymyoglobin to the 'reaction medium' (results not shown). No effect was observed following addition of NaNO₃ or KNO₂. Conversely, addition of NMMA to malate–glutamate-supplemented mitochondria approximately doubled the O₂ uptake (Figure 1B).

These results indicated that NO[•] modulated the mitochondrial respiration; this view was supported by the effects mediated by the substrates and inhibitors of NOS, the lack of effect of either NO₃[−] or NO₂[−] and the reversibility of the inhibition by oxymyoglobin. This final observation also ruled out the likelihood that ONOO[−], a highly oxidizing species formed during the diffusion-controlled reaction of O₂^{•−} with NO[•] (NO[•] + O₂^{•−} → ONOO[−]; $k = 2 \times 10^{10}$ M^{−1} s^{−1} [28a]), contributed to the irreversible damage of a respiratory chain component. Furthermore, the inhibition of the respiratory rates of mitochondria supplemented with L-Arg was similar in States 4 (when the production of O₂^{•−} by mitochondria was high) and 3 (when the production of O₂^{•−} was negligible), and the concentrations of ONOO[−] reported to affect the mitochondrial respiratory chain [29] are far higher (mM levels) than the putative build-up of this species under our experimental conditions [10–40 pmol of ONOO[−] × (min · mg protein)^{−1}; C. Giulivi, unpublished work].

The rates of NO[•] production by intact mitochondria and the inhibition of the respiratory rates were dependent upon the metabolic state in which the mitochondria were maintained. The levels of NADPH, NO[•] production and respiratory rates were evaluated in mitochondria incubated under a variety of conditions (State 1, with endogenous substrate; State 2, with ADP; State 3, with substrate plus ADP; State 4, with substrate; State 3u, with substrate plus uncoupler; Table 1). Under these experimental conditions, L-Arg transport into mitochondria was not altered by the different metabolic states because it does not require respiratory energy and is unaffected by the presence of

Figure 1 Dependence of the respiratory rate of mitochondria in State 4 on L-Arg and NMMA concentrations

The O₂ uptake of intact purified mitochondria was evaluated upon supplementation with 5 mM glutamate–0.5 mM malate (○) or 10 mM succinate (●) in the presence of L-Arg (A) or NMMA (B). The O₂ uptake was measured using 1 mg protein/ml suspended in 'reaction medium' as described in the Materials and methods section.

Table 1 Oxygen uptake, NO[•] production and NADPH content of mitochondria under different metabolic states

Rat liver mitochondria (RCR = 5.5 ± 0.5 and P/O number = 1.7 ± 0.4 with succinate) were incubated under the different conditions in 'reaction medium'. The O₂ consumption of mitochondria (1 mg of protein) was measured at 35 °C in the presence of 10 mM succinate (State 4), substrate plus 0.3 mM ADP (State 3), substrate and 0.5 ng of FCCP/mg protein, or substrate and 50 nmol of Ca²⁺/mg of protein (State 3u), no substrate (State 1), no substrate plus ADP (State 2) or with or without L-Arg. The rate of NO[•] production was followed by the controlled oxidation of oxymyoglobin in the presence of L-Arg. The concentration of L-Arg (0.1–0.3 μ mol/mg protein) used in these experiments was 100-fold higher than the K_m of mtNOS for L-Arg [18], sufficient to sustain a constant production of NO[•]. Other experimental conditions are explained in the Materials and methods section. N.D., not detectable; nd, not determined.

	Oxygen uptake (nano-atom gram O/min per mg protein)		NO [•] production (nmol NO [•] /min per mg protein)	[NADPH] (nmol/mg protein)
	– L-Arg	+ L-Arg		
State 4	20 ± 2	12 ± 2	1.6 ± 0.4	8 ± 2
State 3	105 ± 9	60 ± 6	1.2 ± 0.2	6 ± 1
State 1	7 ± 1	5.6 ± 0.2	1.3 ± 0.1	6.0 ± 0.6
State 3u	105 ± 12	96 ± 9	N.D.	n.d.
State 2	5 ± 2	6 ± 2	0.08 ± 0.2	n.d.

Table 2 Effect of L-Arg on mitochondrial functional parameters

Oxygen uptake of purified mitochondria (2.5 mg) in 'reaction medium' with or without 0.3 μ M L-Arg/mg protein was measured polarographically. The ATP synthesis was evaluated by measuring the amount of ATP in aliquots taken at different time points during State 3. The ATP synthesized was evaluated at the end of State 3.

Mitochondrial functional parameter	— L-Arg	+ L-Arg
Oxygen consumption (nano-atom gram O/min per mg protein)		
10 mM Succinate	20.1 $\pm$ 0.2	12.9 $\pm$ 0.3
10 mM Succinate + 0.2 mM ADP	133 $\pm$ 3	73 $\pm$ 1
ATP synthesis (μ mol ATP formed/min per mg protein)	0.24 $\pm$ 0.02	0.13 $\pm$ 0.05
ATP synthesized (μ mol ATP/mg protein)	1.25 $\pm$ 0.05	1.21 $\pm$ 0.07
RCR	6.6 $\pm$ 0.1	5.7 $\pm$ 0.1
P/O number	1.80	1.74
ATPase activity* (μ mol ATP consumed/min per mg protein)	0.22 $\pm$ 0.01	0.23 $\pm$ 0.01

* ATPase activity was measured using toluene-permeabilized mitochondria (0.2 mg/ml) incubated in 'reaction medium' supplemented with 8.5% (v/v) poly(ethylene glycol) (molecular mass 8 kDa)/100 μ M NADPH/1 mM CaCl_2 , in the absence (— L-Arg) and presence of 80 μ M L-Arg (+ L-Arg).

uncouplers [30]. Mitochondria maintained in States 1, 3 or 4 had high levels of NADPH (6–8 nmol NADPH/mg protein; [25,26]) and exhibited significant rates of NO^* production. Under high NADPH levels, the respiratory rates were inhibited by 20% (State 1), 40% (State 4) and 43% (State 3) respectively, in the presence of L-Arg. Conversely, mitochondria maintained in States 3u or 2 had undetectable levels of NADPH [25,26], negligible production of NO^* and insignificant inhibition of O_2 uptake (Table 1).

Although the levels of NADH, NADPH, NAD^+ and NADP^+ were identified and quantified by HPLC, it was not possible to distinguish whether NADH or NADPH was the electron donor for the NO^* production in intact mitochondria, for both levels were modulated similarly by the different metabolic states (results not shown). Given that NOSs (and their homologous enzyme, NADPH-cytochrome *P*-450 reductase) require NADPH for enzymic catalysis, it could be expected that the activity of mtNOS is also sustained by the level of NADPH. The respiratory rate was inhibited only when a significant production of NO^* occurred, the latter probably sustained by endogenous NADPH, the specific electron donor for NOS. Under our experimental conditions, the energy-dependent transhydrogenase, isocitrate: NADP^+ oxidoreductase (EC 1.1.1.42; [31]), could be considered as the main source of NADPH because uncoupling conditions [overload of Ca^{2+} or carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP)] that facilitate the electron flow from NADPH to NAD^+ [31] affected both the levels of NADPH and the rate of NO^* production.

The effect of NO^* production on oxidative phosphorylation was studied in mitochondria supplemented with L-Arg (Table 2). The rate of ATP synthesis in intact mitochondria was decreased by 50% in the presence of L-Arg (Table 2), similar to the decrease in the respiratory rates of mitochondria maintained in either States 3 or 4 (compare Tables 1 and 2). No significant changes were observed in either the RCR or P/O ratio (Table 2). The total amount of ATP synthesized in intact mitochondria at the end of State 3 (Table 2) and the rate of ATP consumption in permeabilized mitochondria (Table 2) were unaffected by L-Arg addition, indicating that NO^* was not modulating the O_2 consumption and ATP synthesis through direct inhibition of the ATPase.

Figure 2 Dependence of oxygen consumption by intact mitochondria in State 3 on oxygen concentration in the presence of L-Arg and NMMA

The respiratory rate of intact coupled mitochondria in 'reaction medium' supplemented with 10 mM succinate, 0.3 mM ADP (Δ) was evaluated polarographically. Additions of 5 μ M ($\circ$) or 0.1 mM ($\bullet$) L-Arg or 0.1 mM NMMA ($\blacktriangle$) were performed after succinate addition (before ADP). Other experimental conditions are described in the Materials and methods section.

Effect of NO^* on the oxygen dependence of respiration and cytochrome oxidase activity

The dependence on O_2 concentration of the respiratory rate in tightly coupled mitochondria oxidizing succinate in the presence of ADP (State 3) was observed at O_2 concentrations below 15 μ M (Figure 2). The O_2 concentration at half-maximal respiratory rate ranged from 2 to 3 μ M. In the presence of L-Arg (at concentrations equal to, and 20 times higher than, the reported K_m of mtNOS for L-Arg; [18]), the rates of O_2 uptake decreased significantly at all O_2 concentrations. Oxygen concentrations at half-maximal respiratory rate were 18 and 40 μ M, at low ([L-Arg] = K_m) and high concentrations of L-Arg ([L-Arg] = $20 \times K_m$) respectively. Conversely, addition of NMMA to intact mitochondria increased the O_2 uptake by 40–50% of the control values at low O_2 levels; the O_2 concentration at half-maximal respiratory rate in the presence of NMMA was 1.2 μ M.

Given the reactions of NO^* with cytochrome oxidase in *in vitro* systems [32,33], it is possible that NO^* modulated O_2 consumption by inhibiting cytochrome oxidase. This possibility was evaluated by monitoring the dependence of cytochrome oxidase activity on O_2 concentrations in mitochondria supplemented with either L-Arg or NMMA (Figure 3). The effects of substrates or inhibitors of NOS on cytochrome oxidase activities were similar to those observed for the respiratory rate in State 3 (compare Figure 2 with Figure 3). The O_2 concentrations at half-maximal cytochrome oxidase activity were 0.9, 5 and 45 μ M with NMMA, endogenous and exogenous L-Arg respectively.

Although the use of O_2 electrodes has been criticized in the past as being inappropriate for detecting O_2 concentrations below 5 μ M [34,35], the apparent K_m for O_2 of cytochrome oxidase experimentally determined in State 3u (below 0.5 μ M), and the ratio of the apparent K_m under coupled and uncoupled conditions (equal to 10), were found to be comparable with those that had been reported previously for isolated mitochondria using different, more sensitive, methodologies (phosphorescence quenching [36], 'respirograph' [37] and visible spectroscopy [38]). Our results were experimentally supported by the linear response of the digital oxygraph to O_2 concentrations from 0.1 μ M to 250 μ M (see the Materials and methods section).

Figure 3 Dependence of cytochrome oxidase activity in mitochondria on oxygen concentration in the presence of L-Arg and NMMA

The activities of cytochrome oxidase in mitochondria were measured polarographically. Mitochondria suspended in 'reaction medium' plus succinate were supplemented with NO[•] (○), 0.3 μmol L-Arg/mg protein (●) or 0.3 μmol NMMA/mg protein (▲). After 3–5 min of incubation, antimycin was added, followed by ascorbate and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine to evaluate cytochrome oxidase activity. The cytochrome oxidase activities were evaluated during the first 2 min. Other experimental details are described in the Materials and methods section.

Figure 4 Eadie-Hofstee plot of respiratory rates and cytochrome oxidase activities in the presence of L-Arg and NMMA

The respiratory rates (○) and cytochrome oxidase activities (●) were taken from the data in Figures 2 and 3. The data for each experimental condition (i.e. control, + L-Arg, or + NMMA) of both respiratory rates and cytochrome oxidase activities were fitted to a single line.

When the experimental data from Figures 2 and 3 (the O₂ uptake of mitochondria in State 3 and cytochrome oxidase activity) from a given experimental condition were plotted in an Eadie-Hofstee plot, it was possible to fit them to a single line (Figure 4). This indicated that both the O₂ consumption and cytochrome oxidase activity were affected similarly by NO[•], i.e. inhibited by L-Arg but stimulated by NMMA (Figure 4). The unchanged *V*_{max} values (34 ± 4 and 36 ± 4 nmol O₂/min per mg protein in the absence and presence of L-Arg respectively, and 38 ± 4 nmol O₂/min per mg protein with NMMA addition) and the different *K*_m(app) values indicated competitive inhibition kinetics, in which NO[•] inhibited the respiratory rate, competing

Figure 5 Nitrate and nitrite production by SMPs

(A) Nitrate and nitrite concentrations were evaluated in succinate-supplemented SMPs (0.4 mg/ml), incubated as described in the Materials and methods section, using the assay based on the Griess reaction. (B) SMPs maintained in 'reaction medium' under optimal conditions for NOS activity were incubated with (●) or without (○) 10 mM succinate. Aliquots were taken every 5 min for a 30 min period. Nitrate and nitrite were monitored as described in the Materials and methods section.

with O₂ for cytochrome oxidase, and thereby increasing the apparent affinity of cytochrome oxidase for O₂. Given the difference in the *K*_m(app) for O₂ in energized and uncoupled mitochondria found in this and other studies [*K*_m(app) for O₂ of mitochondria in State 4 is 0.5–0.9 μM, and in State 3u 0.04–0.1 μM [36,37,39]], as well as the higher O₂ concentration required for half-maximal activity in isolated cells, perfused heart and *in vivo* heart [38], it could be hypothesized that these differences can be attributed to an endogenous production of NO[•] under *in vivo* conditions.

The competitive inhibition by NO[•] of cytochrome oxidase could be explained by considering NO[•] as an alternative substrate of cytochrome oxidase in which NO[•] is catabolized to product(s) [32,33]. Production of NO₃⁻ and NO₂⁻ (evaluated by the assay based on the Griess reaction) was obtained with substrate-supplemented SMPs incubated under optimal conditions for NOS activity (Figure 5). SMPs were chosen in preference to intact mitochondria to avoid possible secondary reactions of these products with components of the mitochondrial matrix. The production of (NO₃⁻ + NO₂⁻) showed a linear dependency on protein concentration (Figure 5A) during approximately the first 10 min of incubation (Figure 5B). Under these conditions, the rate of NO[•] production by SMPs was 11 ± 1 nmol NO[•] × (min · mg protein)⁻¹ (evaluated by measuring the oxidation of metmyoglobin). This indicated that about 70% of NO[•] was recovered as (NO₃⁻ + NO₂⁻) in succinate-supplemented SMPs (Table 3), whereas the rest (30%) could be accounted for as unreacted NO[•] or other NO[•]-derived products, not detectable by

Table 3 Production of nitrate and nitrite by SMPs

SMPs were incubated as described in the Materials and methods section and, where indicated, with the additions shown: 10 mM succinate, 10 μ M antimycin and 1 μ M SOD. Aliquots were taken every 5 min for a 30 min period, and the levels of ($\text{NO}_3^- + \text{NO}_2^-$) were evaluated using the nitrate reductase–Griess reaction as described in the Materials and methods section. The rates of ($\text{NO}_3^- + \text{NO}_2^-$) production were calculated from the linear dependence of ($\text{NO}_3^- + \text{NO}_2^-$) concentrations with time.

Additions	$+ d(\text{NO}_3^- + \text{NO}_2^-)/dt \text{ nmol} \times (\text{min} \cdot \text{mg protein})^{-1}$
None	0.6 ± 0.2
Succinate	8.0 ± 0.5
Succinate + antimycin	11.0 ± 0.3
Succinate + antimycin + SOD	2.3 ± 0.1

the Griess assay. In the absence of succinate, the rate of ($\text{NO}_3^- + \text{NO}_2^-$) was decreased to $0.6 \pm 0.2 \text{ nmol} \times (\text{min} \cdot \text{mg protein})^{-1}$ (Table 3), probably attributable to the slow conversion of NO^* to $\text{NO}_2^-/\text{NO}_3^-$ in air-saturated, aqueous solutions.

Addition of antimycin (an inhibitor of the respiratory chain between cytochromes *b* and *c*₁) to succinate-supplemented SMPs resulted in an increase (37.5%) in the rate of ($\text{NO}_3^- + \text{NO}_2^-$) production (Table 3). 80% of the ($\text{NO}_3^- + \text{NO}_2^-$) production by antimycin- and substrate-supplemented SMPs was sensitive to micromolar amounts of superoxide dismutase (SOD). The higher rate of ($\text{NO}_3^- + \text{NO}_2^-$) production in antimycin-supplemented SMPs, and the significant inhibition by SOD, may indicate an increased production of ONOO^- (favoured by the enhanced production of $\text{O}_2^{\cdot -}$ during ubisemiquinone autoxidation in the presence of antimycin) which, in turn, decays to NO_3^- ($\text{ONOO}^- + \text{H}^+ \leftrightarrow \text{HONOO} \rightarrow \text{NO}_3^- + \text{H}^+$).

Under physiological conditions, it can be estimated that the formation of ($\text{NO}_3^- + \text{NO}_2^-$) by the succinate dehydrogenase–cytochrome *b* segment in intact mitochondria is almost negligible [9% of the total ($\text{NO}_3^- + \text{NO}_2^-$) production, and 6% of the NO^* formation in mitochondria, calculated as the SOD-insensitive rate of NO_x^- production corrected by the steady-state concentration of ubiquinol without antimycin]. It follows that most (60–65%) of the NO^* produced by mitochondria would be catabolized by the cytochrome *c* $\rightarrow \text{O}_2$ segment, in which cytochrome *c* oxidase plays a major role. This is supported by the haemoprotein– NO^* interaction found in this study (Figure 5), and in others using either purified enzyme [32,33,40,41] or synaptosomes [9]. However, inhibitors of cytochrome *c* oxidase (KCN, Na_3N or Na_2S) failed to show a decreased rate of NO_3^- formation in substrate-supplemented SMPs because these compounds were also effective inhibitors of nitrate reductase at the same concentrations as those required to inhibit cytochrome oxidase.

Biological Significance

This study demonstrates that NO^* produced by mitochondria has important implications for the metabolism of cellular energy. In this regard, the inhibition of cytochrome oxidase by NO^* may result in respiratory rate values between the two physiological limits, i.e. the oxygen uptake in States 3 and 4, modulating the production of ATP to best suit the changing needs of the cell in a dynamic milieu.

The mechanism by which NO^* inhibits O_2 uptake can be understood in terms of a reaction between NO^* (60–65% of the total NO^* produced) and cytochrome oxidase, yielding ($\text{NO}_3^- + \text{NO}_2^-$) as final products. Other species such as N_2O (nitrous

oxide) and NO_2 (nitrogen dioxide), known to be produced during the interaction of cytochrome oxidase with NO^* [32,33], probably were also produced but not detected by the Griess assay. It is not clear how cytochrome oxidase is involved in the production of ($\text{NO}_3^- + \text{NO}_2^-$); however, two molecular mechanisms may be involved: (i) the formation of NO_2^- during the reduction of cytochrome oxidase by NO^* [32], and/or (ii) formation of NO_3^- from NO^* in a reaction analogous to that of oxymyoglobin or oxyhaemoglobin with NO^* ($[\text{Fe}^{\text{II}}\text{OONOCu}] \rightarrow [\text{Fe}^{\text{III}}\text{Cu}] + \text{NO}_3^-$) [42]. Either mechanism could be presumed to operate through the binuclear centre, a process which have a fundamental bearing on the activity of cytochrome oxidase, especially given Wikstrom's proposal [43] that the binuclear centre is critically involved in proton pumping. Transient depolarizations of intact mitochondria observed following the addition of either pure NO^* gas [14] or NO^* endogenously produced (C. Giulivi, unpublished work) may provide further support for the involvement of the binuclear centre in these mechanisms.

Following the work of Chance and Williams, it is well-established in both isolated mitochondria and in cells that an increased ATP utilization can increase ATP synthesis, respiration and substrate oxidation, simply by providing the substrates (ADP and P_i) for oxidative phosphorylation, and mass action stimulates the respiratory chain back to substrate oxidation. Several factors have been proposed to regulate respiration, including Ca^{2+} mobilization, proton leak, adenine nucleotide carriers, oxygen supply and shuttling of ATP. Cytochrome oxidase might be a site for allosteric regulation, because it operates far away from equilibrium (in contrast with the first two phosphorylating sites), has significant control over respiration rate in isolated mitochondria and cells, and contains multiple tissue-specific units with no known catalytic function [44]. However, no significant physiological effectors of cytochrome oxidase were known. Of the available ligands (NO^* , CN^- , N_3^- , HCOOH , S^{2-} and CO), NO^* is the only one that fulfils all the requirements for this role. It is produced at a steady rate by a specific enzyme (mtNOS), is generated close to the target site (cytochrome oxidase) and is formed at levels (10–220 nM; see Appendix 1) which effectively inhibit the respiratory rate (K_i 6–10 nM, obtained by replacing the calculated concentrations of NO^* in the slopes of the Eadie–Hofstee plots; see Appendix 2). As a consequence of this high affinity, the cytotoxic role of NO^* observed in other systems could be explained as a sustained inhibition of cytochrome oxidase by high concentrations of NO^* , such as those achieved by activated macrophages, which would favour a decline in ATP formation and probably lead to cell death.

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APPENDIX 1

Calculation of the steady-state concentration of NO[•]

In order to determine these levels of NO[•], the steady-state concentration of NO[•] was calculated according to eqn. (A1):

$$-d[\text{NO}^{\bullet}]/dt = k[\text{NO}^{\bullet}][a_3] \quad (\text{A1})$$

using $k = 4 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ [45], and in consideration of the fact that approx. 2% of total cytochrome oxidase (experimentally determined to be approx. 0.2 nmol haem a_3 /mg protein) was

reduced. The latter assumption was confirmed by calculating the concentration of reduced cytochrome *c* oxidase from the following equation:

$$V_{\text{max}} = \text{TN}[a_3^{2+}] \quad (\text{A2})$$

where TN (turnover number) is given by $k_1 \times K_m$ ($k_1 = 2 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$; [45]), and K_m was determined experimentally, as described in the text.

APPENDIX 2

Although CO may serve as an intercellular messenger [46–48], its affinity for cytochrome oxidase is similar to (or lower than) that of O₂ [49], indicating that (i) the CO concentrations have to be higher than the $p\text{O}_2$ in a particular tissue (above 10 μM) to exhibit a regulatory role, and (ii) even at these high concentrations, CO

would be rapidly quenched by other haemoproteins with higher affinities for CO (e.g. haemoglobin and myoglobin have 250–300-fold and 25–50-fold higher affinities respectively for CO than for O₂).