Rapid reduction of nitric oxide by mitochondria, and reversible inhibition of mitochondrial respiration by nitric oxide

Vilmanté BORUTAITÉ and Guy C. BROWN*

Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

Nitric oxide (NO) inhibited the respiration rate of mitochondria isolated from rat heart at sub-micromolar concentrations of NO. The inhibition was rapidly and completely reversible, indicating that NO does not damage mitochondria. The sensitivity of respiration to NO depended on the oxygen concentration, substrate type and respiratory state of the mitochondria, consistent with NO competing with oxygen at cytochrome oxidase. Mitochondria catalysed a rapid rate of NO breakdown, which was greater in the absence of oxygen and was partly inhibited by cyanide and azide, suggesting that at least part of the NO breakdown was due to reduction of NO by cytochrome oxidase. The rapid rate of this breakdown suggests that mitochondrial breakdown of NO may be significant physiologically.

INTRODUCTION

Nitric oxide (NO) has the dual roles of intercellular messenger and cytotoxic agent [1–4]. NO has also been implicated in a range of pathologies, which in the heart include damage due to ischaemia, sepsis and endotoxaemia [4–6]. One of the major mechanisms of NO cytotoxicity has been thought to be damage to mitochondrial iron–sulphur centres, causing irreversible inhibition of mitochondrial respiration [2]. However, the levels of NO that have been used experimentally to cause this type of damage to iron–sulphur centres are very high relative to the levels of NO likely to occur *in vivo*.

NO has recently been found to reversibly inhibit cytochrome oxidase [7] and the respiration rate of isolated skeletal muscle mitochondria [8], brain nerve terminals [7] and cultured cells [9], and to cause a decrease in the mitochondrial membrane potential in isolated brain and liver mitochondria [10] and liver cells [11]. This inhibition of cytochrome oxidase by NO has been suggested to be an important means of controlling respiration both physiologically and pathologically [12]. Constitutive isoforms of NO synthase are present in muscle fibres of heart [13] and skeletal muscle [14] and might be involved in regulating contraction [13,14]. Inhibition of NO synthase affects cardiac muscle function in myocytes *in vitro* [13,15], in isolated, perfused heart [16] and in heart *in vivo* [17]. Stimulation of NO production in isolated heart and skeletal muscle has been reported to inhibit tissue respiration, whereas inhibition of NO production stimulates respiration [18,19]. However, the conditions under which NO inhibits mitochondrial respiration are unclear. We show here that the sensitivity of respiration rate to NO depends on the oxygen concentration, substrate type and respiratory state.

NO is known to be produced by various isoforms of NO synthase, but the mechanisms by which NO is broken down are less clear. Within the blood NO is rapidly oxidized by oxyhaemoglobin to nitrate, but cells and tissues in the absence of blood still rapidly break down NO by unknown mechanisms [20]. There is some evidence that isolated cytochrome oxidase [21] and cytochrome oxidase within cultured cells [22] metabolizes NO in anoxic conditions, but at an unknown rate. In this paper we show that mitochondria catalyse rapid breakdown of NO, and at least part of this breakdown is due to reduction of NO by cytochrome oxidase.

EXPERIMENTAL

Mitochondria from Wistar rat hearts were prepared by standard procedures of differential centrifugation [23] in a medium containing 30 mM Tris/HCl, 160 mM KCl, 10 mM NaCl, 5 mM EGTA, pH 7.7 (2 °C). The protein concentration was determined by the biuret method.

Mitochondrial respiration and NO concentration were measured simultaneously at 37 °C in a 1 ml incubation chamber fitted with both a Clark-type oxygen electrode and an NO-selective electrode (described in [7]). The incubation buffer contained 30 mM Tris/HCl, 125 mM KCl, 10 mM NaCl, 5 mM KH_2PO_4, 1.5 mM $MgCl_2$, 3 mM EGTA (pH 7.2), plus respiratory substrates (5 mM succinate + 1 μM rotenone, or 1 mM pyruvate + 1 mM malate). State 3 was obtained by adding 1 mM ADP. The mitochondrial concentration used in this study was 0.5 mg/ml protein. Concentrations of inhibitors used are indicated in the figure legends. The rates of mitochondrial respiration were corrected for a low rate of oxygen diffusion into the vessel, by measuring the rate of oxygen diffusion into the vessel after addition of inhibitors of the respiratory chain.

Rates of NO breakdown were estimated from the NO-electrode trace after the addition of 1 μM NO to the medium. The desired oxygen concentration (measured by oxygen electrode) was reached either by perfusing with nitrogen gas (in the absence of mitochondria) or waiting for respiration to decrease the oxygen to the desired level. In one set of experiments (shown in Figure 3, top panel) the oxygen level in the incubation chamber was maintained at zero by supplementing the medium with 10 units/ml glucose oxidase and 10 mM glucose to consume all the oxygen. Control experiments showed that this system had no significant effect on NO decay, and NO did not inhibit glucose oxidase over the range of NO concentrations used. NO-saturated water was prepared as described [7] and the concentration of NO in NO-saturated water was taken to be 2.0 mM.

RESULTS

We investigated the effect of NO on heart mitochondrial respiration rate in state 4 and state 3 by measuring oxygen consumption and NO concentration in the incubation medium simultaneously. State 4 was originally defined as the state of

* To whom correspondence should be addressed.

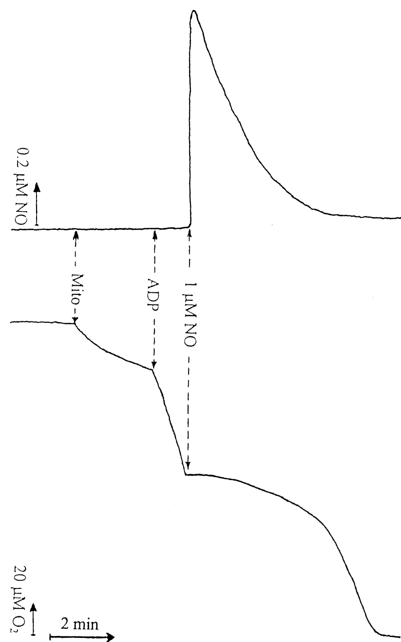
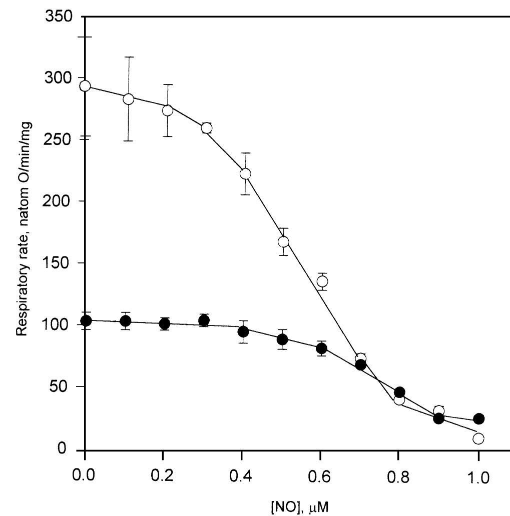


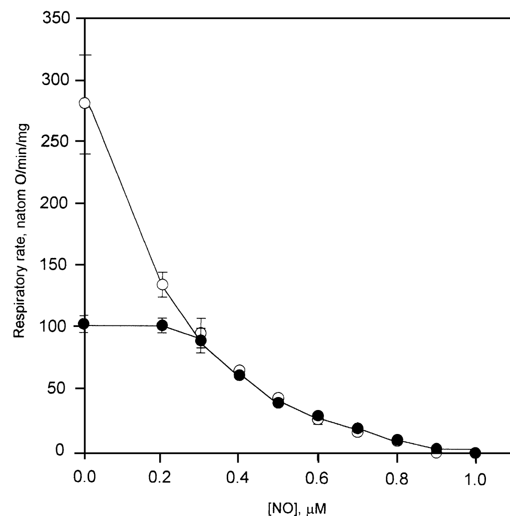
Figure 1 Inhibition by NO of mitochondrial respiration in state 3

The upper trace is from a nitric oxide electrode and the lower trace from an oxygen electrode. At the indicated times 0.5 mg/ml mitochondria was added to the electrode vessel, followed by 1 mM ADP and 1 μM NO. The oxygen trace flattens off at the end due to anaerobosis.

minimal phosphorylation after added ADP has been phosphorylated, but we shall use the term here to refer to the closely analogous steady state in the presence of respiratory substrate but the absence of added ADP; state 3 will refer to the state of maximal phosphorylation after the addition of excess ADP. Mitochondrial oxygen consumption was immediately inhibited by exogenously added NO and increased again as the concentration of NO decayed (Figure 1). Figure 2 shows the dependence of mitochondrial respiration rate on NO concentration during the decay of added NO, with succinate as substrate and either high (top panel) or low (middle panel) oxygen tension, or with pyruvate as substrate at low oxygen tension (bottom panel). In all conditions the sensitivity of respiration rate to NO was lower in state 4 than in state 3. The inhibition of state 3 respiration by NO was more effective at low oxygen levels (52–65 μM O_2; Figure 2, middle panel) than at high (126–168 μM O_2; Figure 2, top panel): the estimated $K_{0.5}$ values for NO were 0.18 μM ($\pm$0.04 S.E.M.) and 0.57 μM ($\pm$0.04) respectively with succinate. Respiration on pyruvate was somewhat more sensitive to NO (estimated $K_{0.5}$ 0.07$\pm$0.004 μM NO at approx. 60 μM O_2).

To determine whether NO can inhibit mitochondrial respiration irreversibly we incubated mitochondria with NO for an extended period. Aliquots of NO were added approximately every 30 s to maintain the concentration of NO in the medium at about 1 ($\pm$0.2) μM for 15–18 min, and the respiration rate was then measured after the NO level had decayed to zero. However, even after long incubation under such conditions the inhibition of mitochondrial respiration by NO was completely reversible: with succinate as substrate the state 3 respiration rate in the control (preincubated without NO for 15 min) was 336$\pm$14 natom of O/min per mg of protein and 355$\pm$9 natom of O/min





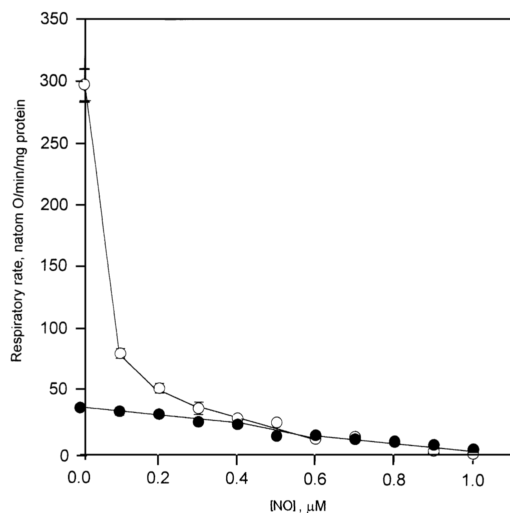


Figure 2 Relationship between NO level and mitochondrial respiration rate in state 4 or state 3

NO (1 μM) was added to mitochondria either respiring on succinate at two different oxygen levels: 126–168 μM O_2 (top panel) or 52–65 μM O_2 (middle panel), or respiring on pyruvate with 52–65 μM O_2 (bottom panel). The dependence of state 4 (●) and state 3 (○) respiration rates on the measured NO concentration during the decay of NO level is plotted. Each point represents the mean$\pm$S.E.M. for three independent preparations of mitochondria.

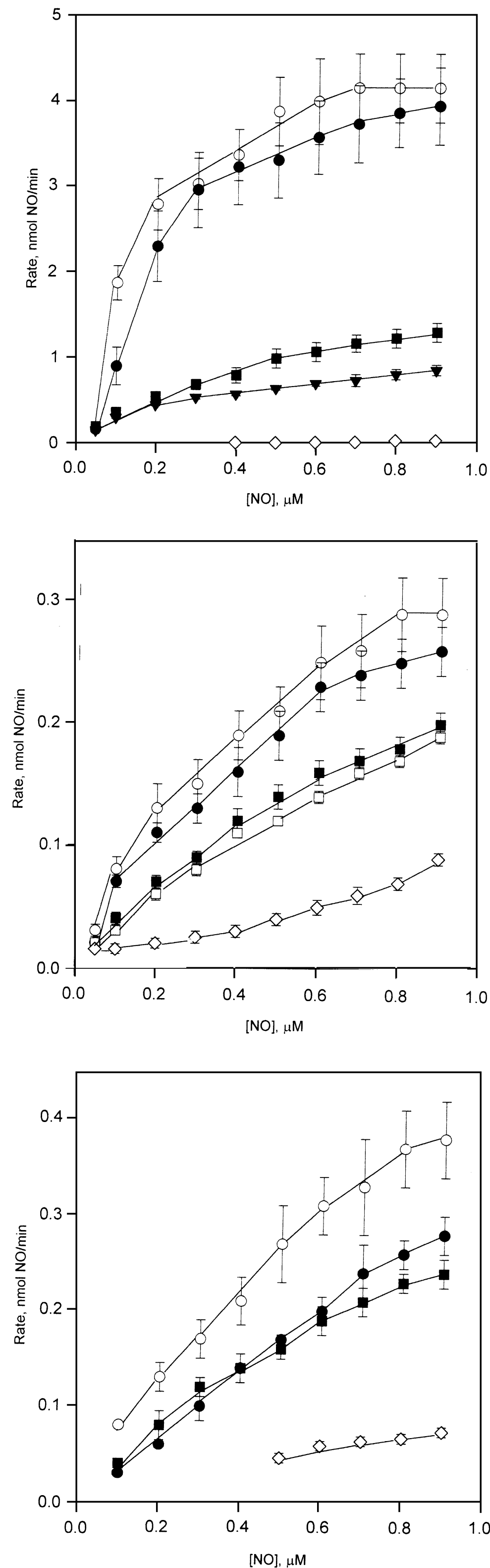


Figure 3 The dependence of the rate of NO decay on the NO concentration

(Top panel) NO decay rate at zero oxygen tension with succinate; (middle panel) NO decay rate at 52–65 μM O_2 with succinate as respiratory substrate; (bottom panel) NO decay rate at 52–65 μM O_2 with pyruvate+malate as substrate. Symbols in the top panel: ◇ no mitochondria; ○, plus mitochondria, no inhibitors; ●, plus malonate; ■, plus azide; ▼, plus cyanide. Symbols in the middle panel: ◇, no mitochondria; ○, plus mitochondria, no inhibitors; ●, plus malonate; ■, plus azide; □, no substrate. Symbols in the bottom panel: ◇, no mitochondria; ○, plus mitochondria, no inhibitors; ●, plus rotenone; ■, plus azide. Inhibitors (5 mM malonate, 10 mM NaN_3, 1 mM KCN or 1 μM rotenone) were added before NO. The *y*-axis rate refers to the rate of NO breakdown within the 1 ml vessel. Mitochondria were added to give a concentration of 0.5 mg/ml protein. Each point represents the mean ± S.E.M. for three independent preparations of mitochondria, except data for NO decay rate without mitochondria where the mean of two repeats is given.

per mg after incubation with NO; with pyruvate as substrate the rates were 274 ± 18 in the control and 260 ± 13 after incubation with NO. Increasing the concentration of free calcium in the medium to 1 μM also had no effect on the reversibility of NO inhibition. State 3 respiration rate in the control was 232 ± 33 natom of O/min per mg of protein, and after incubation with NO it was 217 ± 33 natom of O/min per mg, with pyruvate as substrate.

We also tested whether mitochondria can catalyse NO breakdown. NO was added to the incubation medium either with or without mitochondria (Figure 1), and the dependence of the rate of NO breakdown on the level of NO during the decay is plotted in Figure 3. As can be seen, mitochondria markedly accelerate the rate of NO decay at zero oxygen tension (Figure 3, top panel), and at 52–65 μM O_2 (Figure 3, middle panel) when respiring on succinate, as well as when respiring on pyruvate (Figure 3, bottom panel). To assess which mitochondrial processes might be involved in this NO breakdown we tested the effect of omitting the respiratory substrate or adding respiratory inhibitors. Figure 3 (middle panel) shows that omitting the respiratory substrate (succinate) or adding an inhibitor of cytochrome oxidase (azide) partly inhibited the mitochondria-catalysed NO breakdown, whereas an inhibitor of succinate dehydrogenase (malonate) was rather less effective at inhibiting NO breakdown. Similarly in Figure 3 (bottom panel) we can see that with respiration on pyruvate, azide (an inhibitor of cytochrome oxidase) and rotenone (an inhibitor of complex I) cause a partial inhibition of NO breakdown.

In Figure 3 (top panel) the oxygen tension was maintained at zero (measured by the oxygen electrode) by including glucose oxidase and glucose in the medium; this did not increase NO decay in the absence of mitochondria. The rate of NO breakdown by mitochondria was greatly increased at zero oxygen tension, and this rate was more sensitive to inhibitors of cytochrome oxidase (azide and cyanide), suggesting that cytochrome oxidase was responsible for at least part of the NO breakdown. Azide or cyanide lowered the initial rate of NO decay by 70–80% (P < 0.001) at zero oxygen tension and by 30% (P < 0.001) at 52–65 μM O_2 compared with controls (mitochondria respiring on succinate in the absence of inhibitors). Note also that in the absence of oxygen the azide- and cyanide-sensitive rate of NO breakdown is saturated by relatively low levels of NO (half-saturation at roughly 0.1 μM NO in Figure 3, top panel) whereas, in the presence of oxygen, saturation with NO is not evident at the levels used (up to 1 μM NO). Again this suggests that NO is binding in competition with oxygen to a cyanide- and azide-sensitive site.

DISCUSSION

The finding that NO reversibly inhibits heart mitochondrial respiration at sub-micromolar concentrations is not surprising, given previous findings that NO reversibly inhibits respiration in isolated cytochrome oxidase [7], isolated muscle mitochondria [8], isolated nerve terminals from brain [7], cultured astrocytes [9] and isolated skeletal and heart muscle [18,19], and also causes a

reversible decrease in mitochondrial membrane potential in isolated liver and brain mitochondria [10] and in isolated liver cells [11]. The finding here that the NO inhibition is stronger at lower oxygen concentrations was also observed for the NO inhibition of respiration in isolated nerve terminals [7] and suggests that the inhibition is due to NO competition with oxygen at cytochrome oxidase. We also found here that the NO inhibition is stronger in state 3 than in state 4, which might be due a difference in the extent to which cytochrome oxidase limits the respiration rate in these different states, as it has previously been found that the control coefficient of cytochrome oxidase over mitochondrial respiration is higher in state 3 than in state 4 [24]; this might also be the reason for the higher NO sensitivity of respiration with pyruvate as substrate relative to respiration with succinate. This difference in sensitivity to NO in phosphorylating and non-phosphorylating states might be important physiologically in that, for example, a muscle would be more sensitive to NO inhibition of respiration when contracting than when resting in a non-contracting state.

The high sensitivity of heart mitochondrial respiration to NO suggests that the inhibition might occur physiologically or pathologically. A constitutive isoform of NO synthase (ecNOS) is present in both the endothelium and myocytes of heart [13]. The respiration of isolated canine myocardial muscle is inhibited by bradykinin, which causes physiological NO release from the myocardial endothelium, and this inhibition is reversed by inhibiting NO synthase [19]. Similarly, constitutive isoforms of NO synthase are present in skeletal muscle fibres [14,25], and inhibition of NO synthase increases muscle contraction and oxygen consumption [14,18,25]. Isolated skeletal muscle mitochondria have been reported to have a calcium-dependent NO synthase activity that inhibits mitochondrial respiration [25]. Thus it has been suggested that NO is an endogenous regulator of muscle contraction via its inhibition of cytochrome oxidase [12,25].

Cytokines or endotoxin cause the expression of the inducible form of NO synthase in cardiac myocytes, and subsequent high levels of NO inhibit heart muscle function [5]. This disfunction might also be due to NO inhibition of cytochrome oxidase. Expression of the inducible form of NO synthase in cultured astrocytes has been shown to reversibly inhibit cellular respiration at cytochrome oxidase [9].

The NO inhibition of mitochondrial respiration is entirely reversible, suggesting that NO cannot be directly involved in irreversible damage to mitochondria. This contrasts with the commonly expressed view that NO damages mitochondria by reacting with iron–sulphur centres in complex I, complex II and aconitase [2]. However, when treating brain synaptosomes with 1 μM NO we found no evidence of damage to mitochondrial iron–sulphur centres as detected by EPR or irreversible inhibition of respiration [26]. Thus if iron–sulphur centres are damaged after exposure of cells to NO, the damage may be indirect, for example owing to NO inhibition of catalase [27] and a subsequent build up of H_2O_2. NO reacts rapidly with superoxide to produce peroxynitrite, which has been suggested to mediate the toxic effects of NO [3,4,6]. Mitochondria are a major source of superoxide, and inhibition of cytochrome oxidase should greatly stimulate superoxide production [8], which should then react with NO to produce peroxynitrite. However, the fact that we saw no irreversible inhibition of respiration by NO does not favour this mechanism of NO toxicity.

Mitochondria catalysed NO breakdown apparently by two separate mechanisms: the first was inhibited by respiratory inhibitors and saturated by low levels of NO, whereas the second mechanism was insensitive to respiratory inhibitors and was not saturated by 1 μM NO. Both mechanisms were stimulated in the absence of oxygen and thus presumably involved reductive rather than oxidative reactions, which excludes the involvement of oxygen, superoxide and oxyhaemoglobin. The first mechanism is likely to be mediated by cytochrome oxidase because it is inhibited by cyanide and azide, which are relatively specific inhibitors of cytochrome oxidase, and by the absence of substrate and thus the absence of electron supply to cytochrome oxidase. In the absence of oxygen the cyanide- and azide-inhibitable rate of NO breakdown was half-saturated at about 100 nM NO (Figure 3, top panel), which is comparable to the concentration of NO required to half inhibit respiration at low oxygen levels (Figure 2, middle panel). This again is consistent with the NO breakdown being mediated by cytochrome oxidase.

It has previously been shown that isolated cytochrome oxidase can reduce NO to N_2O (measured by mass spectroscopy) in anoxic conditions with ascorbate as substrate, but at an unknown rate [21]. More recently it was shown that Chinese hamster ovary cells and mitochondria isolated from these cells apparently mediated NO breakdown, and this breakdown was inhibited by cyanide [22], again implicating cytochrome oxidase in NO breakdown. However, NO was measured by line-broadening of the EPR signal of a coal derivative, fusinite, which also responds to oxygen, and thus the experiments could only be conducted in the absence of oxygen. It has been seriously proposed that cytochrome oxidase evolved from NO reductase (a bacterial enzyme that reduces NO to N_2O) on the basis of the homology of the most primitive bacterial forms of these enzymes [28]. The identity of the second mechanism(s) of NO breakdown, not sensitive to cyanide and azide, is unclear.

We can make a crude estimate of whether the mitochondrial catalysed rate of NO breakdown might be significant *in vivo*. The mitochondria decreased the half-life of NO to about 1 min over the concentration range 0.1–1 μM NO when the mitochondrial concentration was 0.5 mg/ml protein and the oxygen concentration was 52–65 μM O_2. The rate of NO breakdown was proportional to mitochondrial concentration, thus with a mitochondrial concentration *in vivo* of roughly 50 mg/ml mitochondrial protein the expected half-life of NO would be about 0.6 s if NO breakdown were mediated by the mitochondria alone; this half-life would be substantially lower at lower oxygen concentrations. The measured half-life of NO in perfused heart was 0.13 s [20]. Thus mitochondrial breakdown of NO may well be significant *in vivo*.

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