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Nitric oxide and peroxynitrite cause irreversible increases in the K_m for oxygen of mitochondrial cytochrome oxidase: in vitro and in vivo studies

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Abstract

Mitochondrial cytochrome oxidase is competitively and *reversibly* inhibited by inhibitors that bind to ferrous heme, such as carbon monoxide and nitric oxide. In the case of nitric oxide, nanomolar levels inhibit cytochrome oxidase by competing with oxygen at the enzyme's heme–copper active site. This raises the K_m for cellular respiration into the physiological range. This effect is readily reversible and may be a physiological control mechanism. Here we show that a number of in vitro and in vivo conditions result in an *irreversible* increase in the oxygen K_m. These include: treatment of the purified enzyme with peroxynitrite or high (μM) levels of nitric oxide; treatment of the endothelial-derived cell line, b.End5, with NO; activation of astrocytes by cytokines; reperfusion injury in the gerbil brain. Studies of cell respiration that fail to vary the oxygen concentration systematically are therefore likely to significantly underestimate the degree of irreversible damage to cytochrome oxidase.

Keywords: Cytochrome *c* oxidase; Nitric oxide; Mitochondria; K_m; Oxygen; Peroxynitrite

1. Introduction

Mitochondrial cytochrome oxidase catalyses over 95% of oxygen consumption by the eukaryotic cell [1]. Nitric oxide (NO) is an intercellular signaling molecule that controls many physiological processes in the body [2]. Nanomolar levels of NO inhibit cytochrome oxidase by competing with oxygen at the enzyme's heme–copper active site [3,4]. This raises the K_m for oxygen of cellular respiration into the physiological range of oxygen tension [5]. This effect is readily reversible and may be a physiological control mechanism in cells [6]. Relief of enzyme inhibition is characterised by conversion of the NO into nitrite [7]; this nitrite formation may be one of the major means of removing NO from inside cells, in order to turn off its signaling effects or reduce its toxicity [8]. The biochemical [9] and cellular physiological [10] consequences of the effect of NO inhibition of cytochrome oxidases have recently been reviewed.

Although persistent long-term, inhibitory effects are seen on other mitochondrial enzymes (e.g. complex I, NADH dehydrogenase, Ref. [11]), it has generally been assumed that when the NO is removed from the solution, cytochrome oxidase enzyme activity completely returns to normal. However, overproduction of NO in many pathophysiological conditions is associated with production of peroxynitrite, the product of the reaction of NO with superoxide [12]. We recently showed that peroxynitrite irreversibly inhibits the turnover of purified cytochrome oxidase, an effect largely mediated via an increase in the K_m for oxygen [13,14]. These effects are only detectable in a high-resolution respirometer where the low K_m's for oxygen of this enzyme can readily be measured [15] and are therefore not detectable in studies where the oxygen

Abbreviations: DTPA, diethylenetriaminepentaacetic acid; PTIO, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; TMPD, (*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine hydrochloride)

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concentration is kept at many times greater than those found in the tissue in vivo [16].

We therefore studied a number of conditions in vitro and in vivo where there is an increase in the concentration of reactive nitrogen species. We found in these cases that there was an increase in the oxygen K_m, not attributable to the presence of residual NO in the solution. Both NO or peroxynitrite could initiate these long-term irreversible effects.

2. Materials and methods

2.1. Pure enzyme studies

Bovine heart mitochondrial cytochrome *c* oxidase was purified as described previously [13]. For the purified enzymes, the oxygen K_m was determined using a high-resolution Oroboros® respirometer—for a full explanation of the technique see Ref. [15]. Briefly, this oxygen electrode system has a very low back-diffusion rate for oxygen even at low oxygen tensions. This allows measurements to be made at low rates of oxygen consumption so that a significant number of data points can be collected over the low oxygen tension (micromolar and submicromolar) where the cytochrome oxidase K_m lies. Software corrections are applied for the response time of the electrode and the autoxidation rate (rate of oxygen consumption in the absence of enzyme). The latter is also oxygen concentration-dependent, and the linear oxygen concentration dependence of this rate was used to correct the data over the full range of oxygen concentrations. The corrected data were then fit to a hyperbolic curve to determine V_{max} and K_m using non-linear least squares regression.

2.2. Cell and mitochondrial studies

The cell and solubilized mitochondrial K_m's were measured using an in-house fabricated version of the Oroboros system. The respirometer was a 1.5 ml thermostated glass oxygen electrode chamber with a Micor® stopper to reduce back-diffusion of oxygen. Again, the rate was corrected at all oxygen tensions by subtraction of the background oxygen consumption rate (a combination of back-diffusion and consumption of oxygen by the electrode). Brain-endothelial derived cell line b.End5 (0.65 ml, prepared as in Ref. [17]), was added to a respirometer at a protein concentration of 4.5 mg/ml. The buffer was 134.2 mM NaCl, 20 mM glucose, 20 mM HEPES, 5.3 mM KCl, 4.1 mM $NaHCO_3$, 2mM $CaCl_2$, 0.43 mM KH_2PO_4, 0.33 mM Na_2HPO_4, pH 7.4, 30 °C. The solution was allowed to go anaerobic via respiration on endogenous substrates. A saturated solution of NO (measured at 2 mM) was then pumped in at a rate of 80 nmol of NO per ml buffer per hour (using a World Precision Instruments UltraMicroPump). The measured steady state extracellular NO concentration under these conditions was 6.1 ± 0.3 μM. After 1 h, the pump was turned off and 14 μM of the NO scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) added to remove the remaining NO. The stopper was then removed from the chamber and the solution stirred to raise the oxygen concentration to ~200 μM. At this point, no free NO remained in the solution as judged by an NO electrode. The stopper was replaced and the oxygen consumption rate measured.

Primary cultures of astrocytes were activated by cytokines as described previously for 24 h [18]. The steady state NO concentration following this activation has been measured at 1.15 μM [19]. The cells were then freeze–thawed three times to break them and placed in the detergent lauryl maltoside to solubilize them. Under these conditions, NO is no longer produced by the cellular homogenate; the concentrations of the NO synthase substrates, arginine and NADPH decrease dramatically and free NO drops to undetectable levels.

2.3. Gerbil brain mitochondrial studies

Non-synaptic gerbil brain mitochondria were isolated from the forebrain. Post-ischemic mitochondria were harvested from gerbils subjected to 30 min of ischemia (via bilateral carotid artery occlusion) followed by a 2-h reperfusion period. Sham-operated controls were identically treated apart from the lack of ischemia. For full experimental details of the protocol, see Canevari et al. [20]. The mitochondria were suspended in a respirometer in 100 mM potassium phosphate, 0.015% lauryl maltoside, 20 μM diethylenetriaminepentaacetic acid (DTPA), pH 7.0, 30 °C. Oxygen consumption by cytochrome oxidase was initiated by the addition of 45 μM cytochrome *c*, 300 μM *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine hydrochloride (TMPD) and 20 mM ascorbate.

2.4. Curve fitting

Non-linear regression was used to determine the V_{max} and K_m, fitting plots of *v* vs. *S* to the simple Michaelis–Menten mechanism. In the case of the isolated enzyme, these values reflect the k_{cat} and K_m for cytochrome oxidase. In the case of whole cell oxygen consumption (Fig. 3), the term oxygen p50 is perhaps more appropriate [20], rather than K_m as factors external to cytochrome oxidase could affect this number. To aid comparisons between studies, all the data were analysed over the same substrate fit range (0–20 μM oxygen).

2.5. Statistics

Unless otherwise indicated, all errors are quoted ± 1 S.D. Standard *t*-tests (paired when appropriate) were used to test for significance between control and treated samples.

3. Results

Purified, solubilized, mitochondrial cytochrome oxidase was incubated with saturating concentrations of ferrocytochrome *c*. When the oxygen concentration in the reaction vessel reached 130 μM, micromolar levels of NO were added. Complete inhibition was observed due to the direct

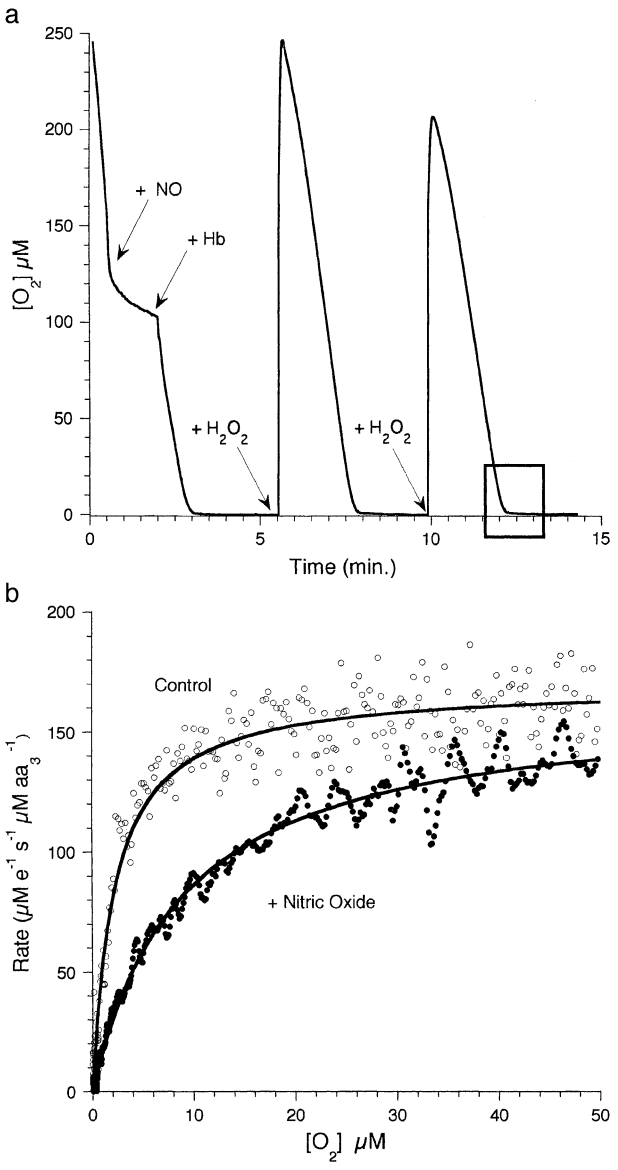

Fig. 1. Method for measuring irreversible effects of nitrogen oxides on cytochrome *c* oxidase kinetic parameters. (a) Cytochrome oxidase (5 nM), in 100 mM K^+ phosphate, pH 7.0, 0.1% lauryl maltoside, 100 nM catalase, 75 units superoxide dismutase, 37 °C, was incubated with 45 μM cytochrome *c*, 300 μM TMPD and 20 mM sodium ascorbate. When the oxygen tension was reduced to 50% of the air-saturated value, NO (10 μM) was added to the solution. NO was removed from the solution by the addition of 100 μM hemoglobin (Hb). Following anaerobiosis, hydrogen peroxide additions were made (450 μM) to inject oxygen into the respirometer. (b) The data in the low oxygen regime (indicated by the box in (a)) were re-plotted as $[O_2]$ vs. rate of oxygen consumption (here illustrated as electron flow through each enzyme molecule per second), allowing V_{max} and K_m to be measured by fitting rectangular hyperbolae.

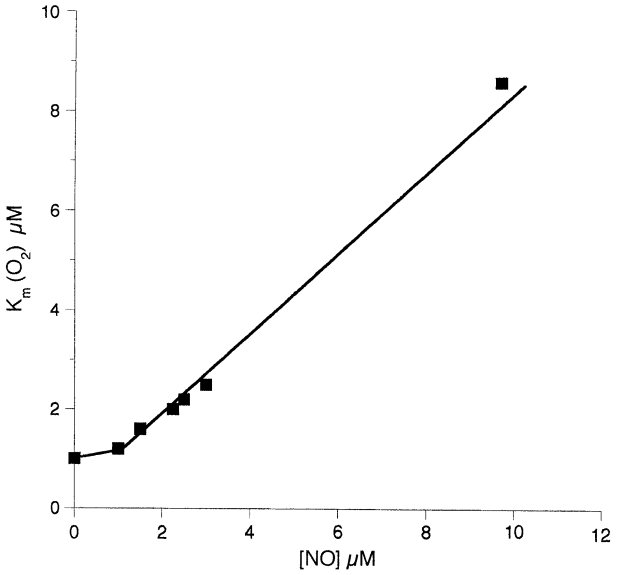

Fig. 2. Irreversible effects of NO on cytochrome oxidase K_m for oxygen. Data obtained from experiments as in Fig. 1, following the addition of different concentrations of NO.

effects of NO. As the NO was removed from the solution, the enzyme activity increased. However, the activity did not return to the 100% uninhibited rate; this effect was exaggerated at low oxygen tensions (<20 μM), suggesting an irreversible effect on the oxygen K_m for cytochrome oxidase.

We therefore performed a systematic study on these irreversible effects of NO on the kinetic parameters of purified cytochrome oxidase. The typical experimental design for these studies is illustrated in Fig. 1. Cytochrome oxidase was incubated with saturating concentrations of ferrocytochrome in an oxygen electrode. At 130 μM oxygen, varying concentrations of NO were added. Hemoglobin was routinely added subsequently to scavenge the NO, although essentially the same effects were used in its absence (so long as enough time had expired so that the NO concentration was reduced to undetectable levels, as judged by separate nitric oxide electrode studies). Catalase and superoxide dismutase were present in the studies described, but there was no noticeable effect if they were omitted. Repeated measurements could be made by re-introducing oxygen via peroxide addition (in the presence of catalase). The data in the low oxygen region (as indicated by the box) were re-plotted as *v* vs. $[O_2]$ plots and analysed for oxygen K_m as described in Materials and methods (Fig. 1b). An increased K_m following NO addition could clearly be seen. The concentration dependence of the NO effect is shown in Fig. 2. NO concentrations lower than 1 μM have negligible effect, but there is already a tripling of K_m by 3 μM.

We confirmed that the raised K_m results from a permanent effect on the enzyme or its immediate environment by measuring the K_m following a number of

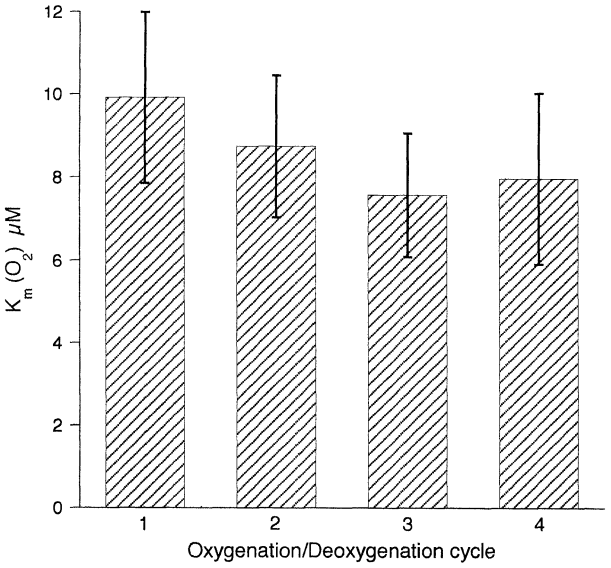

Fig. 3. Irreversible effects of NO on cytochrome oxidase K_m for oxygen. Data obtained from experiments as in Fig. 1 using 10 μM NO. Comparisons made between analysis of the K_m on the first, second, third or fourth deoxygenation cycle. No significant differences were observed.

deoxygenation/reoxygenation cycles. Fig. 3 shows that the increase is permanent and time-independent (i.e. the damage occurs immediately following the NO addition, and not subsequently).

The pattern of irreversible inhibition described here for NO is similar to that we have described previously for $ONOO^-$ [13]. At low concentrations (0–3 μM NO and 0–20 μM $ONOO^-$), the two nitrogen oxides have identical irreversible effects on the V_{max}/K_m ratio, suggesting similar molecular target sites on the enzyme (Fig. 4). At higher concentrations, the effects diverge, and $ONOO^-$ has more effect on V_{max} than NO, for a given increase in the K_m (Fig. 5). This additional damage is likely due to the ability of $ONOO^-$ to degrade the heme and copper prosthetic groups in the enzyme at these higher concentrations [13], and is consistent in general with the more damaging effects of $ONOO^-$ on metalloproteins, when compared to NO [21].

If the nitrogen oxide effects on cytochrome oxidase are irreversible, they should still be observed in cells and in vivo long after the removal of the nitrosative stress. To check this we studied a number of systems undergoing nitrosative stress. It is also possible to observe irreversible increases in the oxygen K_m (p50) in intact cells. In Fig. 6, it can be seen that treatment of an endothelial-derived cell line (b.End5) with low levels of NO for 1 h can permanently raise the p50 for oxygen. Therefore, irreversible increases in the *cellular* oxygen K_m (p50) can be detected following the addition of reactive nitrogen species. It is also possible to

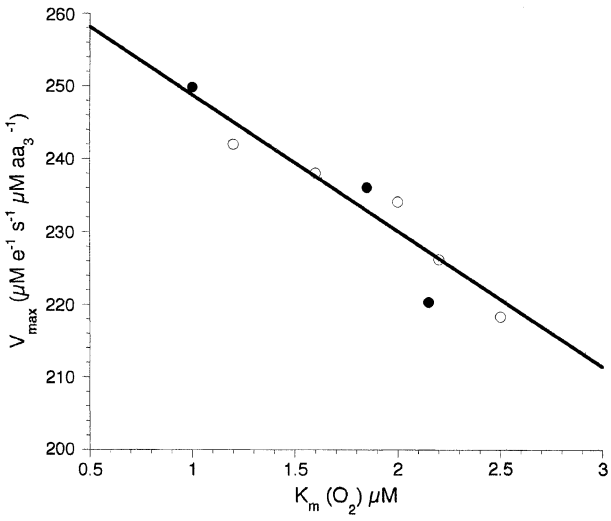

Fig. 4. Comparison of the irreversible effects of low concentrations of NO and peroxynitrite on the cytochrome oxidase kinetic parameters. Conditions the same as in Fig. 1. NO (○) concentration range used, 0–3 μM; $ONOO^-$ (●) concentration range used, 0–20 μM.

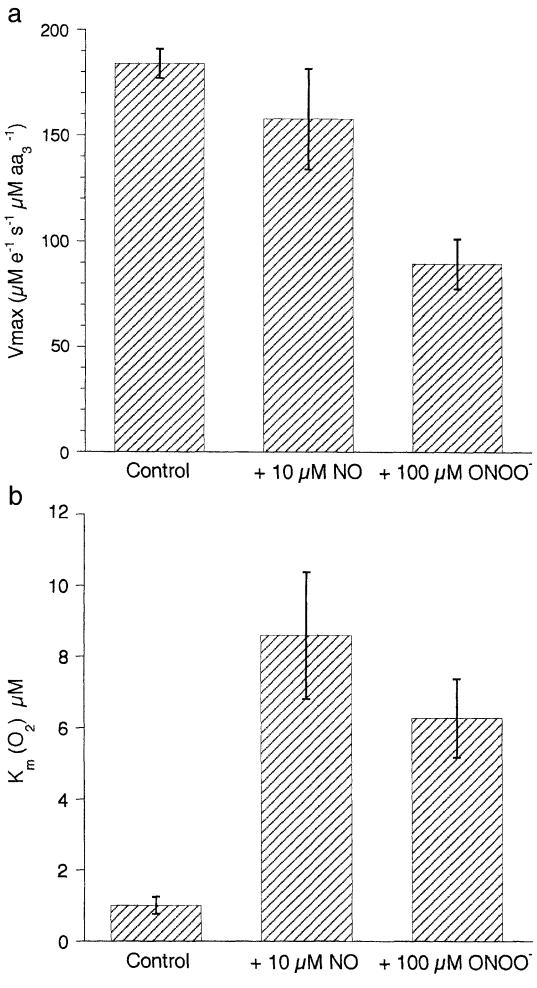

Fig. 5. Comparison of the irreversible effects of high concentrations of NO and peroxynitrite on the cytochrome oxidase kinetic parameters. Multiple experiments ($n=8$) carried out as in Fig. 1 at the indicated concentrations of NO and $ONOO^-$. Effects on V_{max} (a) and oxygen K_m (b). Data plotted as means ± S.D. All data were significantly different from each other ($P<0.05$ or smaller).

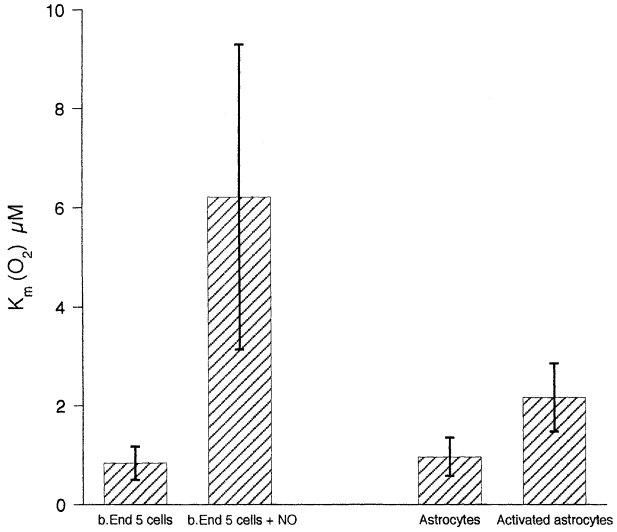

Fig. 6. Irreversible increases in cellular K_m (p50) for oxygen. Experimental details as described in Materials and methods. In b.end5 cells, the K_m (p50) increased from 0.84 ± 0.34 to 6.22 ± 3.08 μM ($n = 19$, $P < 0.001$). Following astrocyte activation, the cytochrome oxidase K_m for oxygen increased from 0.97 ± 0.39 to 2.17 ± 0.69 μM ($n = 6$, $P < 0.01$).

observe these effects when NO is produced endogenously, rather than added by the experimenter. In a previous study, it was shown that the NO produced from the activation of NO synthase by cytokine treatment can competitively inhibit cytochrome oxidase in primary astrocyte cultures [22]. We therefore took astrocytes pre-treated with cytokines for 24 h and solubilized them (a treatment which immediately abolishes significant NO production in the homogenate). The cytochrome oxidase oxygen K_m was then assayed as described in Fig. 1. We detected a small, but significant, long-term irreversible rise in the cytochrome oxidase K_m when compared to control cells (Fig. 6), consistent with the effects of NO or $ONOO^-$ outlined above. The observed K_m increase could not be due to residual low levels of NO production as the addition of the NO scavenger PTIO after solubilization (but before the assay) had no effect on the subsequent K_m measurements.

Irreversible increases in the cytochrome oxidase oxygen K_m can also be observed in mitochondria prepared from animal models of reperfusion injury. Fig. 7 shows that following ischemia-reperfusion injury to the gerbil brain, the V_{max} and K_m for cytochrome oxidase are both perturbed. There is an almost 300% increase in the K_m with a 50% decrease in the V_{max}. This model of reperfusion injury is known to generate nitrosative stress. Whilst we cannot be sure that this is the cause of the observed effect, clearly damage of the same type observed in cytochrome oxidase in vitro can also occur in vivo.

4. Discussion

We have shown that NO can irreversibly raise the oxygen K_m of purified cytochrome oxidase, when directly measured using artificial electron donors and exogenous cytochrome *c*.

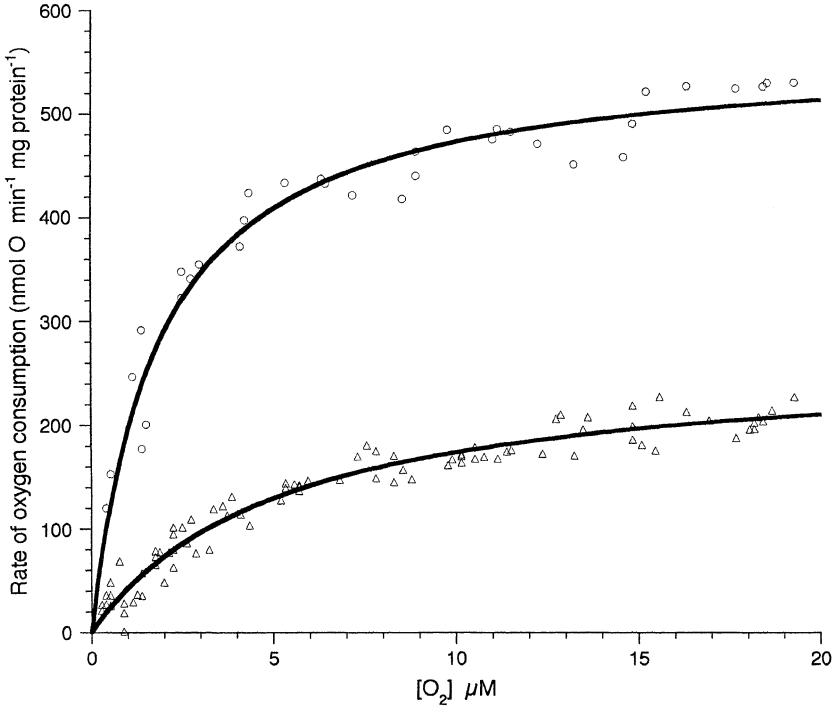

Fig. 7. Irreversible increases in cytochrome oxidase K_m for oxygen in gerbil brain mitochondria following hypoxia-ischemia. Data collected as described in Materials and methods. Values from Michaelis–Menten fit to data (± S.E. from the non-linear regression fit). Control (○): $V_{max} = 563 \pm 3$ nmol O min^{-1} mg $protein^{-1}$; $K_m = 1.89 \pm 0.04$ μM; post-ischemic (△): $V_{max} = 266 \pm 5$ nmol O min^{-1} mg $protein^{-1}$; $K_m = 5.27 \pm 0.10$ μM.

The effect appears kinetically similar to that we have previously reported for peroxynitrite [13], but occurs at lower concentrations. One plausible mechanism for the stronger effect of NO would be if it reacted with an oxygen intermediate to generate a locally high concentration of peroxynitrite in the binuclear center. In this context, it is intriguing that the decomposition of the ferrous-oxy complex of hemoglobin by NO has been purported to occur via a peroxynitrite intermediate [23]. Although, we cannot rule out a direct effect of the N_2O_3 or NO_2, formed during the aerobic breakdown of micromolar NO, the trace concentrations these species reach in the steady state make this unlikely [24].

We have also shown that similar irreversible effects are seen in the p50 for cellular respiration, following prolonged exposure to NO. These findings are likely to have pathophysiological consequences. Nitric oxide and peroxynitrite have both been implicated in the mitochondrial control of apoptotic cell death [25,26] and in a wide variety of pathophysiologies [27]. However, the assays of the effect of these nitrogen oxides on mitochondrial electron transfer in vitro are invariably carried out at unphysiologically high oxygen concentrations where the effects seen in this paper would be completely missed [16,28,29]. Previously it had been thought that the K_m for oxygen of cellular respiration was only affected indirectly, either via the rate of electron entry to cytochrome oxidase from cytochrome *c* or via effects of the mitochondrial proton motive force on internal electron transfer events in the enzyme [30]. It is now clear that direct effects on cytochrome oxidase can reversibly and irreversibly raise the K_m for oxygen. Thus at levels below 1 μM NO, there is very little, if any, irreversible effects of NO on cytochrome oxidase turnover. This is important as it means that physiological levels of NO are unlikely to irreversibly damage the enzyme. However, the effects observed in Fig. 1 are well within the pathophysiological range of NO concentrations as observed in, for example, septic shock [31] or following hypoxia-ischemia [32].

The increased oxygen K_m observed in the purified enzyme is repeated in the cell studies, whether NO is added exogenously, or produced endogenously by activation of NO synthase activity. However, in the cellular studies we cannot control or measure the intracellular NO and peroxynitrite concentrations; therefore we cannot be sure that the increase in K_m is due to the direct NO effects described in Fig. 1. Other factors may come into play. For example $ONOO^-$ formation from NO [13] would also raise the oxygen K_m. Nevertheless, whatever the precise mechanism, it is clear that long-term irreversible effects on cytochrome oxidase can influence cellular respiration and bring the oxygen p50 to levels where there is likely to be a significant perturbation of energy metabolism. In the case of nitric oxide, a reversible effect at nM NO concentrations becomes an irreversible effect at μM levels. Thus reversible inhibition during physiology would become irreversible damage during pathophysiology. The studies on the gerbil brain mitochondria demonstrate that this is a very real possibility. Mitochondria purified from gerbil brains following reperfusion injury have cytochrome oxidase molecules with increased oxygen K_m's. The decrease in the V_{max} described here suggests that other factors may be also at play here; these could include, for example, the removal of the metal centers of the enzyme which occurs at high $ONOO^-$ concentrations [13].

What is the mechanism of NO action? We have previously shown that NO can react with ferrocytochrome *c* to form the nitroxyl anion (NO^-), which can subsequently react with oxygen to form $ONOO^-$ [33]. However, even if all the NO was converted to peroxynitrite by this mechanism the effect would be minimal; 3 μM NO would have to be converted to over 30 μM $ONOO^-$ in solution to have the same effect on V_{max}/K_m. Neither of the stable oxidation products of NO metabolism, nitrite and nitrate had significant effects on cytochrome oxidase turnover. We were unable to inhibit the enzyme with nitrate at any concentration, and the K_i for nitrite inhibition is in the mM concentration range (results not shown).

Although NO interacts with the oxidised Cu_B in the active site [34], under the conditions of the experiments on the purified enzyme described here (fast electron flux at high concentrations of reductants), NO will be primarily interacting with the ferrous heme a_3 site on the enzyme [35]. However, in the cell studies (low to medium electron flux), it is likely that NO is interacting with both Cu_B and heme a_3. Whatever the primary target on the enzyme, the fact that NO and $ONOO^-$ have similar effects at low concentration, suggests that a nitration event is the most likely chemical mechanism for the irreversible damage caused by NO. Bovine heart cytochrome oxidase has 72 tyrosine residues and 18 cysteine residues making a search for potential nitration/nitrosation targets difficult. Even in the key catalytic subunit I, there are seven conserved tyrosine residues between the mammalian and bacterial (*E. coli*) enzymes, likely to play an important role in enzyme function. A number of groups using antibodies to nitrotyrosine, have demonstrated nitration of tyrosine residues [16,36]. Pinning down a specific residue to the functional effects observed here is clearly a non-trivial task. However, we have performed preliminary HPLC/MS data (results not shown) demonstrating nitration of cytochrome oxidase under similar conditions to those described in this paper, suggesting that it may be possible to link structure to function in the future. Intriguingly there are several possible mechanisms by which protein modification could raise the K_m for cytochrome oxidase. It has been shown, for example, that reducing the rate of electron transfer from the low spin heme to the binuclear center [37], or mutating amino acid residue in this "oxygen channel" permanently increase the K_m [38].

Although it is tempting to suggest specific amino acid modifications in the oxidase by reactive nitrogen species, the kinetic data presented here are not wholly consistent with such a model. In the purified enzyme, the *v* vs. *S* plots are good fits to single rectangular hyperbolae throughout

the increase in NO (this paper) or $ONOO^-$ [13] concentrations. A very specific modification of an individual amino acid should lead to two enzyme species, one with a normal and one with a raised oxygen K_m. This would result in biphasic *v* vs. *S* plots, with a higher proportion of the "raised K_m" enzyme at higher inhibitor concentrations. It is therefore possible there are multiple sites on the enzyme (resulting in an *apparent* smooth increase in the K_m) or that the environment sensed by *all* of the enzyme molecules is altered smoothly (e.g. by modification of the lipid bilayer).

In this paper, we have demonstrated a new kind of inhibition of cytochrome oxidase and mitochondrial function, both in vitro and in vivo. In contrast to the in vitro studies, we cannot be sure of the agent responsible in the in vivo studies. However, whatever the molecular mechanism of the increased K_m, these studies have implications for assays of damage to mitochondrial enzymes. The vast majority of these assays are carried out at unphysiologically high oxygen concentrations [14]; conditions in vivo are likely to be such that the increases in oxygen K_m reported here may be critical to cellular function. Therefore, studies that describe in vitro and in vivo effects on cytochrome oxidase activity need to measure the full range of kinetic parameters (including the oxygen K_m) to avoid a significant underestimation of the damage occurring.

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