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Inactivation of nitric oxide by cytochrome *c* oxidase under steady-state oxygen conditions

David C. Unitt, Veronica S. Hollis, Miriam Palacios-Callender, Nanci Frakich, Salvador Moncada *

Wolfson Institute for Biomedical Research, University College London, Cruciform Building, Gower Street, London WC1E 6BT, UK

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ABSTRACT

We have developed a respiration chamber that allows intact cells to be studied under controlled oxygen (O_2) conditions. The system measures the concentrations of O_2 and nitric oxide (NO) in the cell suspension, while the redox state of cytochrome *c* oxidase is continuously monitored optically. Using human embryonic kidney cells transfected with a tetracycline-inducible NO synthase we show that the inactivation of NO by cytochrome *c* oxidase is dependent on both O_2 concentration and electron turnover of the enzyme. At a high O_2 concentration (70 μM), and while the enzyme is in turnover, NO generated by the NO synthase upon addition of a given concentration of L-arginine is partially inactivated by cytochrome *c* oxidase and does not affect the redox state of the enzyme or consumption of O_2. At low O_2 (15 μM), when the cytochrome *c* oxidase is more reduced, inactivation of NO is decreased. In addition, the NO that is not inactivated inhibits the cytochrome *c* oxidase, further reducing the enzyme and lowering O_2 consumption. At both high and low O_2 concentrations the inactivation of NO is decreased when sodium azide is used to inhibit cytochrome *c* oxidase and decrease electron turnover.

1. Introduction

Nitric oxide (NO) is a widespread biological mediator that plays a role in a variety of cell functions [1]. Although the mechanism by which NO is generated by the different NO synthases has been elucidated in detail [2-5], the routes of its inactivation remain controversial. Various possibilities have been suggested, the majority involving interactions with either oxidants [6-10] or haem-containing proteins [11-15]. The existence of an unidentified protein acting as a sink for NO in brain tissue has also been postulated [16]. One potential route of NO inactivation is by cytochrome *c* oxidase, the terminal enzyme in the mitochondrial respiratory chain. This enzyme, which is inhibited by NO, has the capacity to convert NO to nitrite (NO_2^-) when the binuclear centre is predominantly oxidised and the enzyme is in turnover [17-21]. Conversion of NO to NO_2^- has also been demonstrated in cells [22].

We have previously shown, in intact cells, that the inactivation of NO is critically dependent on the NO/oxygen (O_2) ratio and have suggested that at low O_2 concentrations, when cytochrome *c* oxidase is reduced, there is a diversion of NO towards the soluble guanylate cyclase that might explain hypoxic vasodilation [23]. Our previous studies were carried out in an O_2-tight chamber, in which respiring cells consumed O_2 until they reached hypoxia [24]. This imposed constraints on the experimental design, due to the progressive decrease in O_2 concentration in the cell suspension ($[O_2]$) resulting in a short observation period in which the experimental conditions were constantly changing. In order to avoid these limitations we have now developed a system which allows the $[O_2]$ to be controlled and therefore facilitates the study of mitochondrial function in intact cells at a steady $[O_2]$. Here we show in intact cells that NO is converted to NO_2^- at steady-state, physiological $[O_2]$. These results confirm observations that indicate that the cytochrome *c* oxidase plays an important role in regulating endogenous NO concentrations.

2. Materials and methods

2.1. Instrumentation

To enable us to study cells in a stable O_2 environment we have further developed our visible light spectroscopy (VLS) system [23,24] to include a gas headspace within the measurement chamber. By varying the partial pressure of O_2 gas in the headspace we are able to control its concentration in the liquid sample via O_2 exchange between the two phases. The system (Fig. 1) comprises a 10-mm diameter quartz glass chamber surrounded by a water jacket heated to 37 °C, mounted onto a Clark-type O_2 electrode (Rank Brothers, Bottisham, UK). A titanium plunger with a Viton O-ring holds an electrode for NO measurements (ami600, Innovative Instruments, USA) and contains channels to allow gas purging of the headspace and the addition of reagents into the chamber. The chamber holds 1 ml of cell suspension, with a headspace of 0.5 ml. A magnetic, glass-coated stirrer, spinning at 750 rpm, is used to keep the cells in suspension and to help minimise any O_2 gradient within the liquid phase. The

* Corresponding author. Tel.: +44 20 7679 6666; fax: +44 20 7209 0470.
E-mail address: s.moncada@ucl.ac.uk (S. Moncada).

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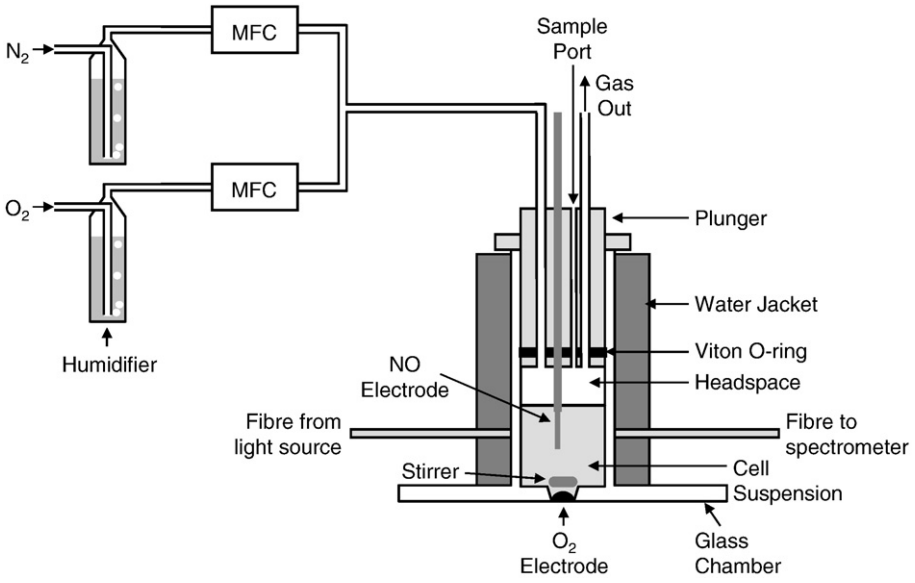

Fig. 1. Diagram of the experimental apparatus. Input gases are humidified and mixed prior to entering the chamber through the plunger, which also holds the NO electrode. There is an O_2 electrode at the base, and optical fibres are flush with the outside of the chamber sides. MFC = mass flow controller.

headspace is continuously purged at a rate of 50 ml·min^{-1} with a mixture of nitrogen (N_2) and O_2 gas. The gases are bubbled through water heated to 37 °C, prior to mixing, in order to humidify them (Fig. 1). The fractions of O_2 and N_2 in the gas mix (O_2^{gas} and N_2^{gas}) are set by two mass flow controllers (1179B, MKS Instruments, Germany). When necessary the gas flow can be turned off and the chamber completely closed so that no headspace remains.

2.2. Measurement and control of oxygen

The [O_2] depends on its relative supply to and removal from the liquid phase. Oxygen is supplied by diffusion from the gas headspace and is removed by cellular respiration, consumption by the O_2 electrode and diffusion back into the headspace. In our system the net flux for a given gas, J_f, from the gas to liquid phase is given by the equation:

$$J_f = m_x(C^* - C) \quad (1)$$

where m_x is the mass transfer coefficient, C is the measured liquid phase dissolved gas concentration and C^* is the concentration in the solution when in equilibrium [25,26]. The O_2 mass transfer coefficient for our system, determined experimentally by generating a step change in the partial pressure of O_2 in the gas phase and measuring the temporal change in [O_2], was found to be $0.0053 \pm 0.0006\ s^{-1}$. We control J_f by changing the ratio of O_2^{gas} and N_2^{gas} and in doing so we can control [O_2]. When J_f matches the cellular oxygen consumption a steady state [O_2] can be maintained. Increasing or decreasing J_f relative to oxygen consumption leads to a raising or lowering of [O_2], respectively.

2.3. Measurement of NO

The NO electrode is calibrated daily by reducing a known quantity of NO_2^- to NO in a reaction with 100 mM H_2SO_4 and 10 mM KI. Typically the electrode sensitivity was over 120 pA·nM^{-1}. The time response of the electrode was measured to be 13.5 ± 1.0 s. There are two main, non-cellular routes for NO to be removed from the liquid phase: diffusion into the gas headspace and NO autoxidation. The NO partial pressure in the gas phase is zero, since the headspace is continuously purged with N_2 and O_2 gases. Therefore, from the equation in Section 2.2, applied to NO gas (with $C^* = 0$), the diffusion of NO out of the liquid phase will be proportional to its concentration in the liquid phase, [NO]. Autoxidation of NO is proportional to [O_2] and the square of the [NO] [27]. In order to assess the contributions of autoxidation and diffusion to the rate of NO removal from the liquid phase in the absence of cells we used the NO donor diethylamine NO (DEANO; $t_{½} = 2$ min) in Hanks-VLS solution (20 mM HEPES, 5.5 mM D-glucose, 5.37 mM KCl, 1.26 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 137 mM NaCl, 4.2 mM $NaHCO_3$, 0.34 mM Na_2HPO_4 and 1% dialysed heat-inactivated foetal bovine serum (HI-FBS)) and measured [NO] as a function of time at different constant [O_2]. After addition of 1 μM DEANO there was an initial, sharp rise in [NO] followed by a more gradual fall as production stopped and NO was removed from the liquid phase (Supplementary Fig. 1). At O_2 concentrations between 10 and 85 μM neither the rate of NO release nor the peak concentration exhibited any significant dependence on [O_2]. The maximum NO concentration reached in these measurements (Supplementary Fig. 1) was higher than that reached in other experiments described in this paper, but was still in the nM range. This indicates that, at the lower concentrations of NO and at the [O_2] used in other experiments in this study, the contribution of autoxidation to the rate of removal of NO is small relative to that of diffusion. Therefore, since autoxidation did not significantly affect the results it was excluded from the analysis.

2.4. Measurement of cytochrome redox states

Spectroscopic measurements are made with a Shamrock 303i spectrometer fitted with a 600 lines·mm^{-1} grating and a charge-coupled device (CCD) camera (Andor Technology, UK). A broadband light source (77501 LOT Oriel, UK) illuminates the liquid phase through an optical fibre that is flush with the outside of the glass chamber. A band-pass optical filter (KG5T, UQG Optics, UK) blocks out unwanted light and heat. Transmitted light is collected by a second fibre, also flush with the outside of the chamber, and passed to the spectrometer and CCD for detection, Fig. 1.

Changes in the redox state of haem a and haem a_3 are derived from changes in the measured optical attenuation, as described previously [28]. For simplification, throughout this paper we refer to changes in the redox states of haems a and a_3 as changes to the redox state of cytochrome *c* oxidase. Briefly, the change in attenuation is converted to a change in redox state through a least-squares fitting of the difference (oxidised minus reduced) absorption spectrum of

cytochrome *c* oxidase in the range 590–615 nm. The difference absorption spectrum is a combination of the difference spectra of haem a (which contributes 80–90% of the total signal) and haem a_3 [24]. In some of our measurements we used sodium azide as an inhibitor of cytochrome *c* oxidase activity that binds only to the oxidized catalytic centre, the species responsible for conversion of NO to NO_2^-. Azide binding to the oxidized haem a_3-CuB causes a blueshift (~2 nm) in the α-peak of the absorption spectrum [29] and it should be noted that this is not accounted for in the fitting procedure. The redox state is expressed as a percentage, with 100% being the fully oxidised state reached after addition of 1 μM myxothiazol to cells at high $[O_2]$ and 0% being the reduced state achieved under closed chamber anoxic conditions, determined separately. Therefore, reference to a percentage increase in the redox state of cytochrome *c* oxidase indicates that it has become more oxidised, whilst a decrease refers to reduction.

2.5. Cell culture and induction of Tet-iNOS-293 cells

We used a tetracycline-inducible human embryonic kidney cell line (Tet-iNOS-293) that has been transfected to stably express nitric oxide synthase upon induction with tetracycline, as described by Mateo et al. [30]. Tet-iNOS-293 cells were cultured in T-175 flasks with phenol red-free Dulbecco's modified eagles medium (DMEM) containing 25 mM D-glucose, 4 mM glutamine, 15 $\mu g \cdot ml^{-1}$ blasticidine, 200 $\mu g \cdot ml^{-1}$ hygromycin B and 10% (v/v) of HI-FBS, as previously described [30]. The cell line produced maximal NO synthase expression after incubation in induction medium (DMEM, 10% HI-FBS, 1.5 $\mu g \cdot ml^{-1}$ tetracycline and 500 μM S-ethylisothiourea (S-EITU)) for 15 h. The addition of S-EITU prevents generation of NO by the NO synthase. Cells were harvested by trypsinisation between 15 and 20 h after induction, centrifuged at 115×*g* for 10 min and re-suspended in Hanks-VLS solution [28] at a concentration of $\sim 5 \times 10^6$ cells $\cdot ml^{-1}$. The cells were placed in a conical flask in a shaking water bath (80 rpm, 37 °C) for 1 h to remove the S-EITU used in the induction medium. They were then centrifuged again and re-suspended at $1–2 \times 10^7$ cells $\cdot ml^{-1}$ in Hanks-VLS solution. At this stage, cell viability, measured by the Trypan Blue exclusion method, was above 95%. The cells were then returned to the water bath where they were kept for a minimum of 1 h more. Post-experimental counting of each cell sample was carried out using a Coulter Counter (Z series, Beckman Coulter, FL) as previously described [28].

L-arginine, S-EITU, tetracycline and trypsin inhibitor were from Sigma. Blasticidin was from Calbiochem. All other tissue culture solutions and media, as well as hygromycin B, were from Invitrogen. DEANO was from Alexis Biochemicals.

2.6. Experimental procedure

The procedure used in each experiment was essentially the same. Cells were prepared in bulk as described in Section 2.5. A 1-ml aliquot of cell suspension was then placed in the chamber and the plunger inserted, after which the gas headspace was continuously purged with 50 $ml \cdot min^{-1}$ N2 and O_2 gas mix. Initially O_2^{gas} was set to 0.5%, which, along with O_2 consumption by the cells, resulted in a rapid fall in $[O_2]$ from an initial value of ~180 μM. When the $[O_2]$ was close to the required concentration, O_2^{gas} was increased so that the O_2 partial pressure in the headspace was high compared to that in cell suspension, resulting in a reversal in the direction of O_2 diffusion. By adjusting O_2^{gas} appropriately it was possible to control the diffusion rate so that it matched the cellular consumption rate, thus achieving a steady-state $[O_2]$. This procedure did not usually take longer than 5 min. Once the required $[O_2]$ was reached, no further changes to O_2^{gas} were made. The cell suspension was allowed to stabilise for a further 5 min prior to the addition of L-arginine. Throughout the whole experiment $[O_2]$, [NO] and cytochrome *c* oxidase redox state were monitored once every second. In some experiments 500 μM sodium azide was added to the cell suspension when the $[O_2]$ reached 100 μM during the initial purging process, while the cytochromes were still predominantly oxidised.

2.7. Data analysis

The area under the curve (AUC) of plots of NO concentration against time was used to quantify NO release in the experiments described in Section 3.3. The AUC was calculated by numerically integrating each NO trace over a period of 15 min, from the time of L-arginine addition, using the trapezoidal method (OriginPro 8, OriginLab, USA), with units of μM · min. All values of NO concentration are given from a baseline taken at the point of addition of L-arginine.

Means ± standard deviations were calculated for quantitative analysis of all data, with the number of samples (n) in each experimental group indicated in the associated text. The two-sample -test was used to determine statistically significant differences between means, with a significance level of $P<0.05$.

3. Results

3.1. Concentration-dependent generation of NO from L-arginine

We investigated the release of NO and its subsequent effect on the cytochrome *c* oxidase redox state and $[O_2]$ in induced Tet-iNOS-293 cells after the addition of different concentrations of L-arginine. The cells were collected as described in Section 2.5. After being placed in the chamber the cells were stabilised at a constant $[O_2]$ of 70.3 ± 2.0 μM ($n = 32$) for 5 min. L-arginine (1–500 μM) was then added, resulting in a concentration-dependent release of NO within seconds from the cells. The amounts of NO released after the addition of 5, 10, 50 and 100 μM L-arginine are shown in Fig. 2A (upper panel). Both the amount of NO released and the time taken for the release to reach a peak were dependent on the concentration of L-arginine added (Fig. 2B and not shown; 8 concentrations, each $n = 4$).

At concentrations of 10 μM L-arginine or greater the release of NO was accompanied by a concentration-dependent reduction in the redox state of cytochrome *c* oxidase (Fig. 2A, centre panel). At lower L-arginine concentrations (<10 μM) no such change occurred. As the [NO] increased (at concentrations of L-arginine >10 μM) there was also a decrease in O_2 consumption by the cells, resulting in an increase in the $[O_2]$ in the chamber (Fig. 2A, lower panel). For all subsequent measurements of the inactivation of NO by cytochrome *c* oxidase 5 μM L-arginine was used, since this generated the maximum concentration of NO that did not affect the redox state of cytochrome *c* oxidase (or O_2 consumption) at 70 μM O_2.

3.2. O_2-dependent generation of NO from NO synthase

We investigated the kinetics of NO release from NO synthase in cells in which mitochondrial respiration was inhibited with sodium azide to prevent NO inactivation and avoid competition for O_2 as a substrate. Cells were placed in the chamber and 500 μM sodium azide was added when $[O_2]$ reached 100 μM during the initial purging process. They were then stabilised at various constant $[O_2]$ for 5 min before the addition of excess (1 mM) L-arginine. The concentration of NO released was recorded and the maximal rate of release, which occurred immediately (within 20–30 s) after L-arginine addition, was found to be $[O_2]$-dependent (Fig. 3). Data were taken on 5 different days. Each data set was corrected for the time response of the NO electrode and fitted to the Michaelis–Menten equation. No account has been taken of NO diffusion into the headspace as this is low during the short measurement period of these experiments. Similarly, $[O_2]$ is assumed to be constant during this time. The NO synthase was found to have a mean apparent K_m for O_2 of 16.1 ± 4.1 μM and a V_{max} of 20.6 ± 5.0 $nM \cdot s^{-1}$ ($n = 5$).

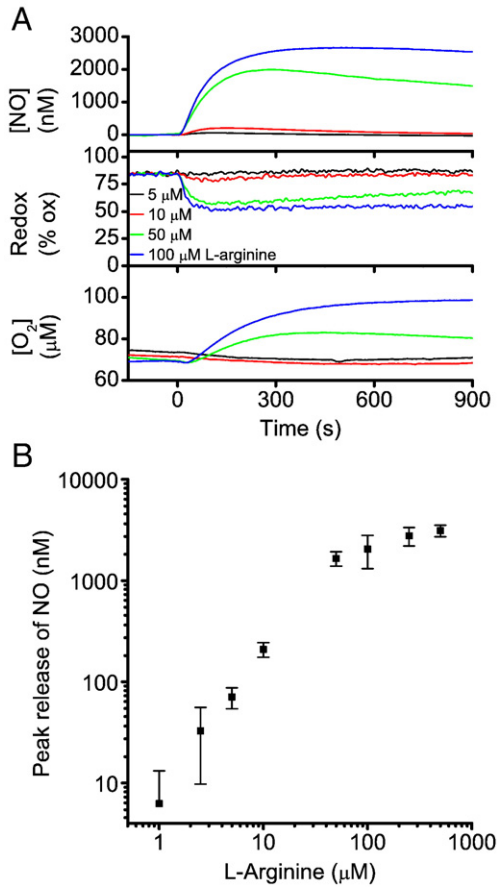

Fig. 2. Concentration-dependent generation of NO from L-arginine. (A) Effect of 5 (black), 10 (red), 50 (green) and 100 (blue) μM L-arginine on release of NO (upper panel), cytochrome *c* oxidase redox state (% oxidised, centre panel) and [O_2] (lower panel) in Tet-iNOS-293 cells. Trace representative of 4 experiments. Prior to addition of L-arginine [O_2] was stabilised at 70.3 ± 2 μM (n = 32). (B) The peak concentration of NO released (± standard deviation) as a function of L-arginine added (n = 4).

3.3. NO inactivation by cytochrome c oxidase at steady-state [O_2]

We have previously demonstrated in an O_2-tight system that NO is inactivated in respiring cells when cytochrome *c* oxidase is in turnover, and that the degree of inactivation decreases as the enzyme becomes more reduced at low [O_2] [23]. Using our present system we carried out experiments in which 5 μM L-arginine was added to cells maintained at either 70 or 15 μM O_2 in the absence or presence of sodium azide.

In cells held at a steady concentration of 70 μM O_2 the cytochrome *c* oxidase was 83.7 ± 1.6% oxidised (n = 8). Addition of 5 μM L-arginine caused the release of NO, which reached a peak after approximately 2 min and then declined (Fig. 4A, upper panel). This concentration of L-arginine had no measurable effect on either the redox state of cytochrome *c* oxidase (Fig. 4A, centre panel) or the [O_2] (Fig. 4A, lower panel). In contrast, in cells held at a steady concentration of 15 μM O_2 the cytochrome *c* oxidase was 77.5 ± 1.8% oxidised (n = 8). The difference in redox state between 70 and 15 μM O_2 was small but significant (P<0.05) and at both concentrations the enzyme was predominantly oxidised. In cells held at concentrations above 15 μM O_2 any change in [O_2] resulted in only a small change in redox state. However, in cells where O_2 fell below 15 μM any further reduction in [O_2] caused a pronounced change in redox state, with the enzyme becoming predominantly reduced. Thus, it is at concentrations at and below 15 μM O_2 that we expect to see large changes in NO metabolism and other mitochondrial activity, as has been suggested by our previous results [23]. In cells held at a steady concentration of 15 μM O_2 the addition of 5 μM L-arginine resulted in the release of NO (Fig. 4B, upper panel), as well as a further, significant (P<0.05), decrease in the redox state of cytochrome *c* oxidase (Fig. 4B, centre panel) to 68.7 ± 5.4% (n = 8), due to inhibition of the enzyme by NO. This inhibition decreased the consumption of O_2, as demonstrated by the increase in [O_2] following the NO release (Fig. 4B, lower panel).

The addition of 500 μM sodium azide at 100 μM O_2 during the initial purging process resulted in a maximal reduction in cytochrome *c* oxidase redox state. At stable [O_2] of both 70 and 15 μM the addition of 5 μM L-arginine caused a greater and more sustained release of NO (Fig. 4C and D, upper panels) than was observed in the absence of sodium azide. However, this release of NO had no further effect on the redox state of cytochrome *c* oxidase, nor did it affect the [O_2] (Fig. 4C and D, centre and lower panels).

The effect of the cytochrome *c* oxidase redox state and electron turnover on NO inactivation was determined by quantifying the amount of NO released upon addition of L-arginine, using the AUC (see Section 2.7) for NO concentration plotted against time. Fig. 5 shows the AUCs at 70 and 15 μM O_2 in the absence and presence of sodium azide. At 70 μM O_2 the AUC was 1.18 ± 0.53 μM·min (n = 8), while at 15 μM O_2 it was significantly higher (P<0.05) at 2.28 ± 0.98 μM·min (n = 8). In the presence of sodium azide, when cytochrome *c* oxidase was not in turnover, the AUCs were significantly higher (P<0.05) than in the absence of sodium azide at both 70 and 15 μM O_2, being 4.98 ± 2.61 μM·min (n = 8) and 6.47 ± 2.25 μM·min (n = 8), respectively. The AUCs determined in the presence of sodium azide at 70 and 15 μM O_2 were not significantly different from one another. Thus, for an L-arginine concentration of 5 μM, the activity of NO synthase had not significantly changed with [O_2], suggesting that, in this case, L-arginine is the limiting factor in NO synthesis.

4. Discussion

We have developed our VLS system to include a gas delivery mechanism so that we are able to control the [O_2] inside the chamber. This has allowed us to investigate the interaction between NO and O_2 at the catalytic centre of cytochrome *c* oxidase under steady-state [O_2] conditions.

We studied the release of NO and the redox state of cytochrome *c* oxidase at 70 μM [O_2] in Tet-iNOS-293 intact cells, in which NO is generated by a tetracycline-inducible NO synthase upon addition of L-arginine. Low L-arginine concentrations (<10 μM) resulted in the release of small amounts of NO without affecting the cytochrome redox state or steady-state [O_2]. However, higher concentrations of L-arginine generated more NO, which led to a decrease in the redox state of cytochrome *c* oxidase and an inhibition of cellular respiration. These results are in agreement with our previous

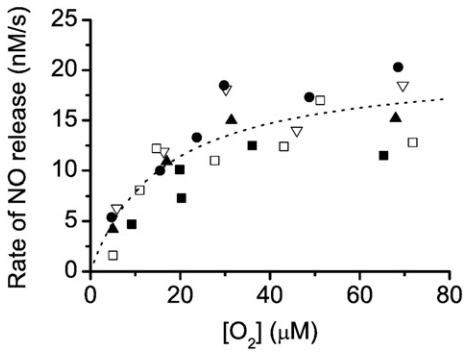

Fig. 3. Initial rate of NO release after addition of 1 mM L-arginine to cells in which mitochondrial respiration has been inhibited. Data were obtained on five different days. The dotted line is a plot of the Michaelis–Menten equation using K_m = 16.1 ± 4.1 μM and a V_{max} of 20.6 ± 5.0 nM·s^{-1}.

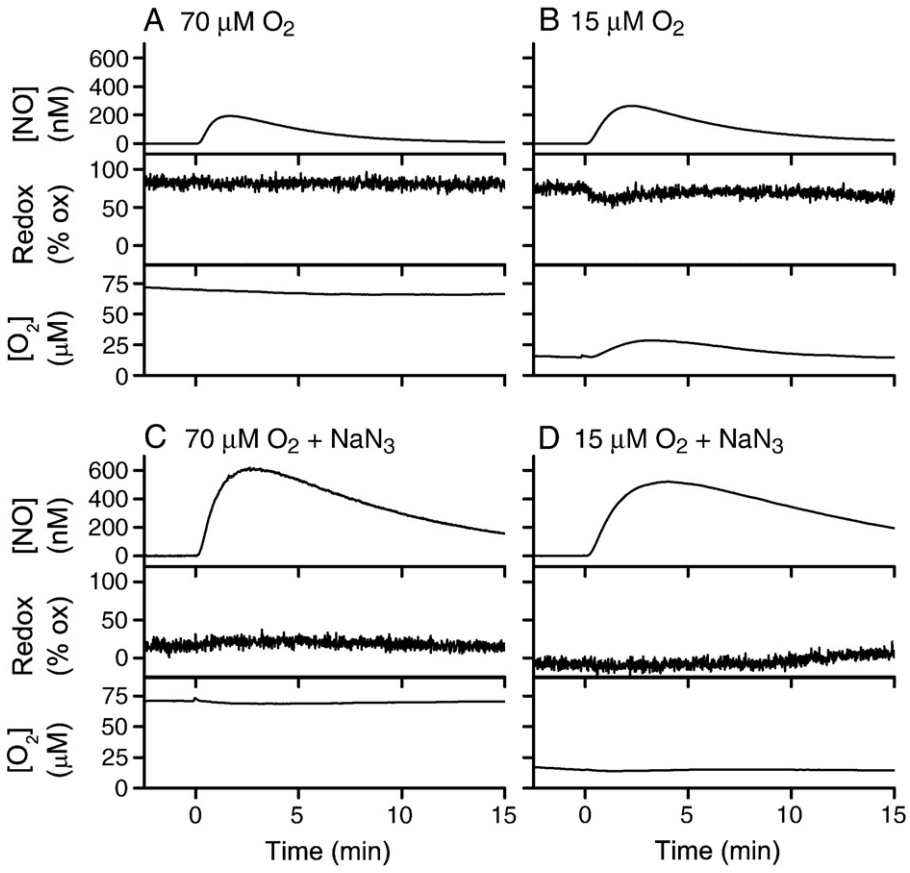

Fig. 4. Effect of [O_2] and sodium azide (NaN_3) on cytochrome *c* oxidase. The measured [NO] (upper panel), cytochrome *c* oxidase redox state (% oxidised, centre panel) and [O_2] (lower panel) are shown for cells at an initial concentration of 70 μM (A, C) and 15 μM O_2 (B, D) in the absence (A, B) and presence (C, D) of 500 μM sodium azide. In all cases 5 μM L-arginine was added at $t=0$ min. Trace representative of 8 experiments.

observations using a closed chamber VLS system, in which we demonstrated that, in the presence of low NO concentrations interacting with the enzyme, cytochrome *c* oxidase can recruit extra capacity to maintain O_2 consumption [28]. Once all the available capacity has been utilised, further increases in [NO] result in the reversible inhibition of cytochrome *c* oxidase. This inhibition leads to a fall in the O_2 consumption rate, which was seen here as a transient rise in the measured [O_2] after addition of L-arginine.

Interestingly, the total amount of NO released as a function of L-arginine concentration in these experiments was much higher than that obtained in our previous experiments with a closed chamber [23]. In those experiments all O_2 was consumed within ~5–10 min. The lack of O_2, which is a substrate of NO synthase, eventually restricted NO production, thus limiting the total amount of NO that could be produced. In our current experiments there is no such limit and NO synthase can continue to generate NO as long as L-arginine is still available.

In order to determine whether the different [O_2] studied in our experiments affected the rate of generation of NO from NO synthase we measured the rate of NO release from cells in which respiration had been inhibited by the addition of sodium azide. In our study application of Michaelis–Menten kinetics gave an apparent K_m of O_2 for NO synthase of 16.1 ± 4.1 μM. The cells were exposed to the different [O_2] for only the period of experiment itself (<20 min), therefore the changes in NO production observed were unlikely to be a result of changes in concentration or expression of NO synthase or other factors that may affect the activity of the enzyme over the longer term. In comparable experiments with RAW 264.7 cells, also subjected to a brief exposure to a given [O_2], a K_m of 31 μM has been reported for murine inducible NO synthase [31]. For cells exposed for longer periods to different [O2] the apparent Km has been measured at 137 μM [32] and estimated to be 14 Torr (~17.5 μM O2) at the cell surface [33]. Experiments in the isolated enzyme [34] and simulations of NO production by induced NO synthase [35] have shown that the apparent K_m depends on the build up of NO, which itself will inhibit the activity of NO synthase. In our experiments cells were incubated in the absence of L-arginine and in the presence of SEITU in order to suppress the production and build up of NO. During the measurement period itself, and after the addition of L-arginine, the highest [NO] reached was not sufficient to inhibit the enzyme [36].

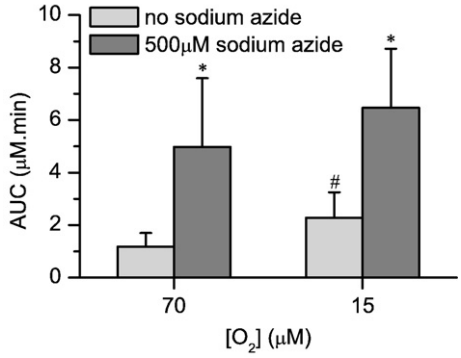

Fig. 5. Effect of [O_2] and sodium azide on NO metabolism. The NO release, quantified as the AUC, calculated over 15 min after addition of 5 μM L-arginine at 70 and 15 μM O_2, in the absence (light grey) and presence (dark grey) of 500 μM sodium azide. (*) Significant ($P<0.05$) difference from the respective control, (#) significant ($P<0.05$) difference from control at 70 μM O_2 ($n=8$).

We have previously demonstrated that the rate of inactivation of NO by cytochrome *c* oxidase is determined by the redox state of the enzyme in turnover [23]. Here we have seen that significantly more NO was released (as quantified by the AUC) from uninhibited cells at 15 μM O_2, where the cytochrome *c* oxidase redox state was 77.5 ± 1.8%, than at 70 μM O_2, where it was 83.7 ± 1.6%, indicating that a more oxidised enzyme inactivates more NO. This result was not due to different amounts of NO being generated at 70 and 15 μM O_2 since cells in which the cytochrome *c* oxidase had been inhibited with sodium azide released the same amount of NO at both [O_2]. At 15 μM O_2 the NO inhibited the cytochrome *c* oxidase, causing a further reduction of its redox state to 68.7 ± 5.4% and inhibition of respiration. This leads to a decrease in the inactivation of NO by cytochrome *c* oxidase, effectively diverting NO towards other targets inside and outside the cell [23] and providing a mechanism whereby the redox state of cytochrome *c* oxidase at low [O_2] may influence physiological parameters such as vasodilation. This is in contrast with studies in which an increase in [NO] at low [O_2] was also observed, but was attributed to the conversion of NO_2^- to NO by either cytochrome *c* oxidase itself [37,38], cytochrome bc_1 (mitochondrial complex III) [39,40] or other cytosolic enzymes [41-43] under hypoxic conditions. Interestingly, a recent study that modelled the metabolism of NO by the oxidised cytochrome *c* oxidase suggested that the rate of this reaction is higher, by an order of magnitude, than other modes of NO inactivation in mitochondria [44].

We then investigated the effect of turnover of cytochrome *c* oxidase on the inactivation of NO. Addition of sodium azide at 100 μM O_2, when cytochrome *c* oxidase is mainly oxidised, stopped its turnover. At both 70 and 15 μM O_2 addition of 5 μM L-arginine resulted in a release of NO that was significantly greater than that released by their respective controls. This indicates that the enzyme, in turnover, plays a major role in the cellular inactivation of NO.

In summary, we have designed a respiration chamber in which the [O_2] can be controlled and have used it to study the inactivation of NO by cytochrome *c* oxidase in intact cells. This system allows us to study the kinetic parameters of enzymes such as NO synthase in vivo. Our results demonstrate that NO inactivation is dependent on both the electron turnover and redox state of cytochrome *c* oxidase, further indicating that this enzyme is an important biological regulator of NO concentrations in cells and tissues.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbabio.2009.12.002.

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