

Cytochrome c oxidase maintains mitochondrial respiration during partial inhibition by nitric oxide

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Summary

Nitric oxide (NO), generated endogenously in NO-synthase-transfected cells, increases the reduction of mitochondrial cytochrome c oxidase (CcO) at O₂ concentrations ([O₂]) above those at which it inhibits cell respiration. Thus, in cells respiring to anoxia, the addition of 2.5 μM L-arginine at 70 μM O₂ resulted in reduction of CcO and inhibition of respiration at [O₂] of 64.0±0.8 and 24.8±0.8 μM, respectively. This separation of the two effects of NO is related to electron turnover of the enzyme, because the addition of electron donors resulted in inhibition of respiration at progressively higher [O₂], and to their eventual convergence. Our results indicate that

partial inhibition of CcO by NO leads to an accumulation of reduced cytochrome c and, consequently, to an increase in electron flux through the enzyme population not inhibited by NO. Thus, respiration is maintained without compromising the bioenergetic status of the cell. We suggest that this is a physiological mechanism regulated by the flux of electrons in the mitochondria and by the changing ratio of O₂:NO, either during hypoxia or, as a consequence of increases in NO, as a result of cell stress.

Key words: Cytochrome c oxidase, Electron turnover, Mitochondrial respiration, Nitric oxide, Redox state

Introduction

It has been proposed that some forms of mitochondrial cell signalling are related to the regulation of the terminal respiratory chain enzyme cytochrome c oxidase (CcO) by nitric oxide (NO) (Moncada and Erusalimsky, 2002). NO is known to inhibit reversibly CcO and hence the rate of mitochondrial oxygen consumption (i.e. mitochondrial respiration; VO₂) (Cleeter et al., 1994; Brown and Cooper, 1994; Schweizer and Richter, 1994). However, it remains to be established whether the interaction of NO with CcO always requires inhibition of respiration in order to be biologically relevant.

It has been observed in cells, isolated mitochondria and living tissues that certain mitochondrial cytochromes appear to accumulate in their reduced forms at O₂ concentrations ([O₂]) well above those at which the mitochondrial respiration becomes inhibited (Wilson et al., 1979; Wilson et al., 1988; Stingle et al., 1996). This phenomenon, the existence of which has been disputed (Wittenberg and Wittenberg, 1985; Chance, 1988; Arthur et al., 1999), has been termed 'pre reduction' or 'early reduction' (Chance, 1988) mainly owing to the fact that in a typical O₂-tight chamber, in which O₂ is depleted progressively over time, reduction of the cytochromes is observed prior to inhibition of respiration. It has also been claimed to be a metabolic adaptation whereby changes in mitochondrial redox states are required to maintain respiration and hence mitochondrial ATP flux as [O₂] decreases (Connett et al., 1990).

Using a system based on visible-light spectroscopy (VLS) we are able to monitor simultaneously in intact cells [O₂], [NO] and VO₂, as well as the redox states of cytochrome b_H from

complex bc₁, cytochromes cc₁ – a combined signal from cytochromes c and c₁ (from complex bc₁), and cytochromes aa₃ from CcO (Hollis et al., 2003). In studies of respiring cells, under conditions in which we can rule out factors cited to explain the phenomenon as artefactual (Chance, 1988), we have consistently observed a reduction in cytochromes cc₁ and aa₃ at [O₂] above those at which respiration becomes inhibited (Hollis et al., 2003; Palacios-Callender et al., 2004). Steady-state and kinetic modelling of the NO-CcO interaction (Mason et al., 2006) has predicted that NO can cause a reduction of the mitochondrial cytochromes without inhibiting respiration across the entire in vivo range of [O₂]. Furthermore, we have demonstrated that this phenomenon is dependent on the presence of endogenous NO and that it has redox signalling consequences (Palacios-Callender et al., 2004; Quintero et al., 2006).

In the present study we have used a well-characterised human cell line transfected with an NO synthase (NOS) under the control of a tetracycline-inducible promoter, in which the production of endogenous NO can be finely controlled (Mateo et al., 2003). We show that increases in the concentration of endogenously-generated NO, within the physiological range, lead to an increase in [O₂] at which a reduction of mitochondrial cytochromes can be observed without inhibition of respiration. We suggest that this is a physiological mechanism resulting from a change in the O₂:NO ratio, either during hypoxia or as a consequence of increases in the generation of NO due to cell stress. The interaction between NO and CcO causes a partial reduction of the mitochondrial respiratory chain, which is compensated for by an increase in

the flux of electrons through the uninhibited NO-free CcO, thus maintaining respiration without compromising the bioenergetic status. On the basis of our results we propose a general mechanism to explain how the NO-CcO interaction leads to this compensation.

Results

Concentration-dependent generation of NO from L-arginine

Following exposure to 1.5 μg ml⁻¹ tetracycline for 15 hours, the tetracycline-inducible cell line Tet-iNOS 293 expressed NOS protein maximally. Addition of exogenous L-arginine (1-1000 μM) at 70 μM O₂ to induced cells respiring to anoxia led to a concentration-dependent generation of NO that was detected extracellularly while respiration and cytochrome redox states were monitored. Fig. 1 shows the peak release of NO, which reached a maximum at 50 μM L-arginine.

Concentrations of L-arginine >10 μM resulted in the generation of NO in sufficient quantities to produce an immediate inhibition of respiration. Further studies were therefore carried out at concentrations of L-arginine ≤10 μM.

Reduction of cytochromes cc₁ and aa₃, and inhibition of respiration at different concentrations of L-arginine

As shown in Fig. 2A, the addition of increasing concentrations of L-arginine (1-10 μM) to respiring cells led to an increased reduction of cytochromes cc₁ at progressively higher [O₂]. A similar effect of L-arginine on the reduction of cytochromes aa₃ and inhibition of respiration was also observed (Fig. 2B). However, for any concentration of L-arginine, inhibition of respiration always occurred at a lower [O₂] than that at which increases in the reduction of cytochromes aa₃ (and cc₁) were detected. This effect of L-arginine was entirely dependent on NO generation, because it was abolished by treatment with the NOS inhibitor S-ethylisothiourea (S-EITU) at 500 μM (Fig. 2C). These effects of NO occurred before its maximum generation from L-arginine (shown in Fig. 1) so that (e.g. following the addition of 2.5 μM L-arginine) the concentrations of NO at which cytochromes aa₃ were reduced and respiration was inhibited were 5.4±2.8 nM and 30.9±7.1 nM (n=6), respectively.

Table 1 shows the [O₂] at which significant (P<0.001) changes occurred in both cytochromes aa₃, and respiration at different concentrations of L-arginine (1-10 μM). Because L-

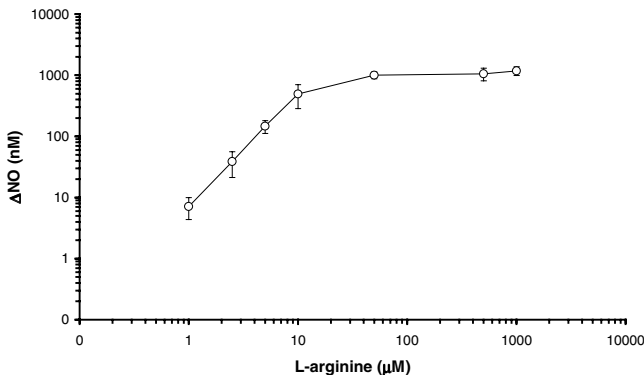


Fig. 1. Peak release of NO after addition of L-arginine to induced Tet-iNOS 293 cells at 70 μM O₂.

arginine was always added at 70 μM O₂ this experimental design imposed an artificial limit so that reduction of cytochromes could not be detected at [O₂] above 65 μM. Consequently, as the concentration of L-arginine increased, the [O₂] at which inhibition of respiration occurred approached that at which the cytochromes became more reduced. These

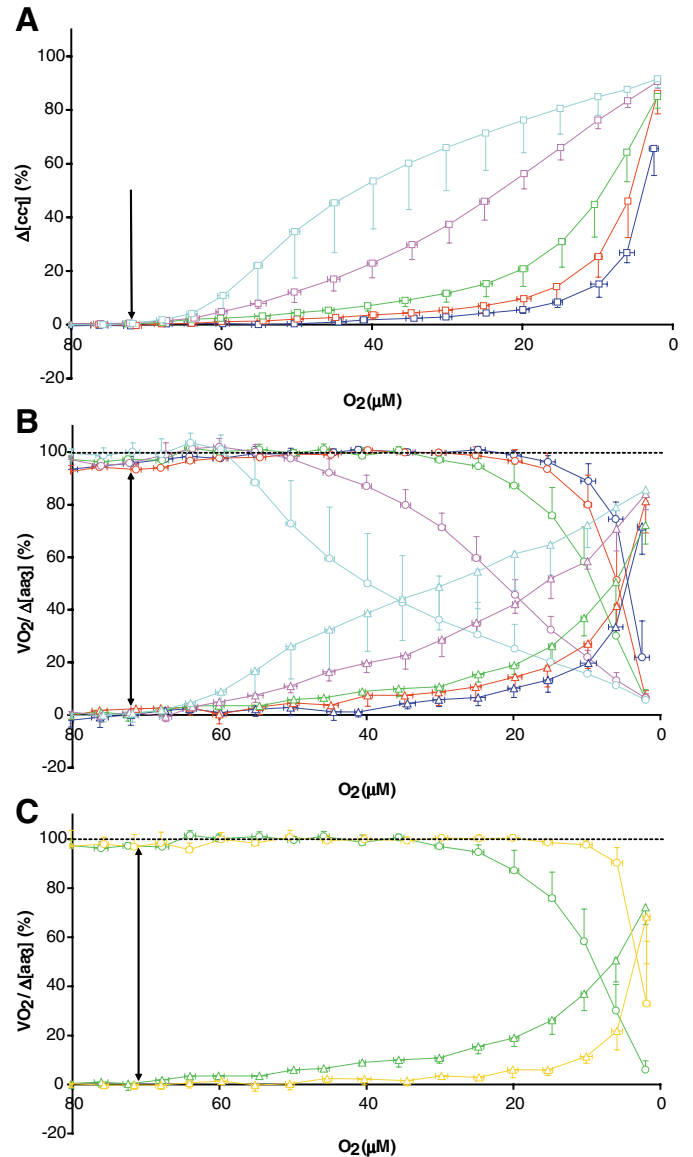


Fig. 2. (A) Changes in reduction of cytochromes cc₁ (squares) upon addition of 0 μM (blue), 1 μM (red), 2.5 μM (green), 5 μM (magenta) and 10 μM (cyan) L-arginine to induced Tet-iNOS 293 cells (n=6 for each concentration of L-arginine). Arrow indicates addition of L-arginine at 72.1±3.1 μM O₂ (n=24). (B) Changes in VO₂ (circles) and reduction of cytochromes aa₃ (triangles) upon addition of 0 μM (blue), 1 μM (red), 2.5 μM (green), 5 μM (magenta) and 10 μM (cyan) L-arginine to Tet-iNOS 293 cells (n=6 for each group). Arrow indicates addition of L-arginine at 72.1±3.1 μM O₂ (n=24). (C) Changes in VO₂ (circles) and reduction of cytochromes aa₃ (triangles) upon addition of 2.5 μM L-arginine to Tet-iNOS 293 cells in the presence (gold) and absence (green) of 500 μM S-EITU (n=6 for each group). Arrow indicates addition of L-arginine at 70.7±4.1 μM O₂ (n=12).

Table 1. Effect of L-arginine on [O₂]_{aa₃} and [O₂]_{VO₂} in induced Tet-iNOS 293 cells

L-arginine (μM)	[O ₂] _{aa₃} (μM)	[O ₂] _{VO₂} (μM)	Cyt aa ₃ at [O ₂] _{VO₂} (%)
0	34.4±1.1	9.8±1.1	19.8±2.8
1	54.5±0.8	15.4±0.3	18.0±6.7
2.5	64.0±0.8	24.8±0.8	15.5±3.0
5	63.7±0.7	45.1±0.8	16.4±3.4
10	64.9±0.6	55.2±0.7	19.5±5.9
0 + S-EITU	30.7±0.9	6.1±0.1	23.2±8.7
2.5 + S-EITU	29.8±0.8	5.9±0.8	21.8±7.7
		Mean ± s.d.	18.8±6.6

The [O₂] at which changes in cytochromes aa₃ ([O₂]_{aa₃}) and VO₂ ([O₂]_{VO₂}) deviate significantly ($P<0.001$) from baseline are shown, as well as the percentage change in cytochromes aa₃ at [O₂]_{VO₂} ($n=6$ per treatment group).

data are presented in Fig. 3, in which the width of the bars represent the [O₂] range in which the cytochromes became more reduced (left side of the bar) and respiration was inhibited (right side of the bar). Assigning 0% reduction to the baseline and 100% reduction to the fully anoxic state (see Materials and Methods), significant inhibition of respiration was observed as cytochromes aa₃ reached around 19% reduction from baseline ($n=42$, see Table 1), regardless of the concentration of L-arginine added. A one-way ANOVA showed that there were no significant differences in this percentage change between groups. The fact that treatment with the NOS inhibitor S-EITU (500 μM) was able to lower the [O₂] at which both increased reduction of the cytochromes and inhibition of respiration occurred (teal bar) – compared with the 0 μM L-arginine group (blue bar) – is evidence of a basal generation of NO from endogenously-produced L-arginine that occurs after the washing out of S-EITU in all the groups (see Materials and Methods).

Addition of 1 μM myxothiazol blocked electron flux through the respiratory chain, and cytochromes aa₃ and cc₁ became fully oxidized, as previously described (Hollis et al., 2003). In this situation, the further addition of 10 μM L-arginine did not change the redox state of either cytochromes

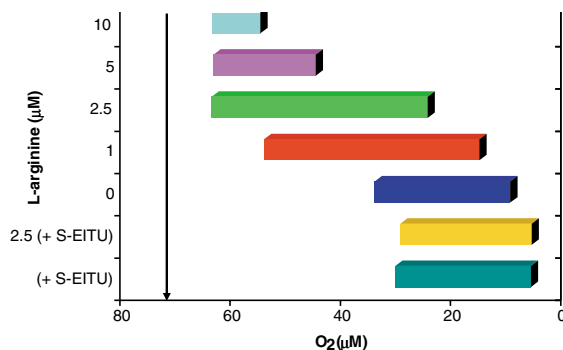


Fig. 3. Graph representing cytochrome reduction and inhibition of VO₂ in induced Tet-iNOS 293 cells upon addition of 0 μM (blue), 1 μM (red), 2.5 μM (green), 5 μM (magenta) and 10 μM (cyan) L-arginine, and 0 μM (teal) and 2.5 μM (gold) L-arginine in the presence of 500 μM S-EITU ($n=6$ for each group). Left and right sides of bars indicate [O₂] at which significant ($P<0.001$) changes from baseline values occur in cytochromes aa₃ and VO₂, respectively. Arrow indicates addition of L-arginine at 71.3±3.4 μM O₂ ($n=30$).

aa₃ or cc₁, in spite of the fact that up to 500 nM NO was generated ($n=6$, data not shown). This demonstrates that the maintenance of respiration during increased reduction of the cytochromes is not caused by a direct effect of NO on cytochrome c in these conditions (see Discussion).

Effect of electron turnover of CcO on the reduction of cytochromes aa₃ and inhibition of respiration

The fact that cytochromes aa₃ can be reduced from baseline values by up to 19% but respiration is still maintained suggests that CcO can increase its activity, i.e. electron turnover (eTN), under the reductive pressure of increasing concentrations of its substrate (reduced cytochrome c), which accumulates as a consequence of the interaction between NO and CcO. The ability of the enzyme to compensate in this way will therefore depend on how close to its maximum capacity it is working. Because of this we investigated whether changes in eTN of CcO affect the [O₂] at which inhibition of respiration occurs. We observed that there is a variation in the steady-state eTN in untreated cells (calculated as described in Materials and Methods). We therefore separated untreated cells into two groups, with an eTN of approximately 50 and 60 electrons per second. We also increased the eTN of CcO artificially using two different concentrations of the electron donors TMPD and ascorbate (180 μM TMPD in 6 mM ascorbate and 300 μM TMPD in 10 mM ascorbate).

Table 2 shows the steady-state values for eTN and the corresponding rate of respiration for all the groups. The [O₂] at which respiration was inhibited was then studied in each group (see Fig. 4). In separate groups of cells in which the same four steady-state eTNs of CcO had been established, L-arginine (2.5 μM) was added and the [O₂] at which respiration was inhibited was studied as before. As shown in Fig. 4, there was a small but significant ($P<0.05$) correlation between eTN of CcO and the [O₂] at which respiration was inhibited in cells without exogenously administered L-arginine. This correlation could be attributable to the formation of basal concentrations of NO from the endogenous pool of L-arginine, as described above. The addition of 2.5 μM L-arginine increased the significance ($P<0.01$), with a greater dependence on eTN. The inset to Fig. 4 shows the effect of eTN of CcO on the decrease in respiration from 100% in cells treated with 2.5 μM L-arginine. As the eTN of CcO increased, so did the [O₂] at which respiration was inhibited, indicating that the enzyme is less able to compensate for inhibition by NO as its eTN increases. The [O₂] at which cytochromes aa₃ became significantly more reduced was not analysed in these experiments, owing to a

Table 2. Effect of various treatments on electron turnover (eTN) and rate of mitochondrial oxygen consumption (VO₂)

Ascorbate (mM) + TMPD (μM)	eTN (e ⁻ sec ⁻¹)	VO ₂ per 10 ⁷ cells ml ⁻¹ (μM min ⁻¹)
0	50.1±1.6	14.2±1.8
0	62.3±2.3	18.7±2.8
6 + 180	79.0±11.8	25.0±1.9
10 + 300	111.5±8.1	32.1±2.3

Induced Tet-iNOS 293 cells were treated with 0 mM ascorbate, 6 mM ascorbate + 180 μM TMPD or 10 mM ascorbate + 300 μM TMPD ($n=3$ per group). Values shown are per 10⁷ cells.

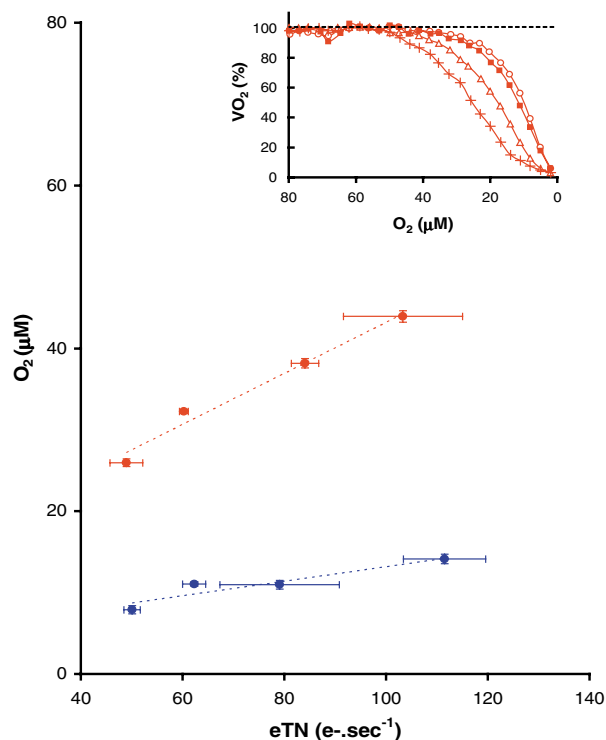


Fig. 4. Effect of eTN of CcO on the $[O_2]$ at which VO_2 becomes inhibited in induced Tet-iNOS 293 cells in the absence (blue) and presence (red) of $2.5 \mu\text{M}$ L-arginine ($n=3$ for each eTN group). Inset shows the changes in VO_2 upon addition of $2.5 \mu\text{M}$ L-arginine at $70 \mu\text{M}$ O_2 for eTN values (in electrons per second) of 49.0 ± 3.2 (open circles), 60.2 ± 0.9 (closed squares), 84.1 ± 2.7 (open triangles) and 103.3 ± 11.7 (crosses).

spectroscopic interference between the cytochrome and TMPD absorption spectra; this was more marked at low O_2 and higher TMPD concentrations.

Discussion

The VLS system allows us to study changes in the mitochondrial cytochrome redox states of cells during respiration (Hollis et al., 2003). Using a cell line stably transfected with a NOS isoform we have developed a model in which nanomolar concentrations of NO can be generated upon the addition of L-arginine. Using this system we now confirm that the endogenously-generated NO can produce, via its interaction with CcO, an increased reduction of cytochromes aa_3 and cc_1 without inhibiting respiration. Inhibition of respiration occurs only when the reduction of the cytochromes reaches a critical point. We have previously shown that this increased reduction allows cell signalling to take place while respiration, and hence energy supply, are not compromised (Palacios-Callender et al., 2004). Now we describe how the maintenance of respiration is achieved by a mechanism in which CcO increases its eTN in order to compensate for an increasing inhibition by NO.

In intact cells CcO does not operate at full capacity. We observe steady-state eTN values of 50–60 electrons per second that can be approximately doubled by the addition of electron donors. Values in the purified enzyme of close to 200 electrons per second or higher have been reported (Sharpe and Cooper,

1998a). This excess capacity of CcO (Letellier et al., 1994) is the basis of the mechanism we now propose. At an $[O_2]$ above that at which the increased cytochrome reduction occurs, the total population of CcO works at a relatively constant steady-state eTN. Upon addition of L-arginine, NO interacts with a fraction of the total enzyme population at the catalytic centre ($a_3\text{-Cu}_B$). As a consequence electron flux through the catalytic cycle is inhibited in this sub-population, resulting in an accumulation of electrons outside the binuclear centre. Because of the thermodynamic equilibrium established within the enzyme ($\text{Cu}_A \leftrightarrow \text{cyt } a \leftrightarrow \text{cyt } a_3\text{-Cu}_B$), electrons accumulate first in cytochrome a and subsequently in cytochrome c. This leads to the observed increase in the reduction of cytochromes aa_3 and cc_1 . As noted in Materials and Methods, the absorption feature associated with the measurement of cytochromes aa_3 is predominantly (80–90%) owing to cytochrome a. Thus, rather than directly reflecting redox events at the catalytic centre, the observed reduction of cytochromes aa_3 indicates that cytochrome a is becoming more reduced as a consequence of the inhibition of CcO by NO. The concomitant accumulation of reduced cytochromes cc_1 occurs as a result of the decreased ability of the inhibited CcO to oxidise cytochrome c and not, as the experiment in which myxothiazol was added demonstrates, owing to a direct reduction of cytochrome c by NO, as has been suggested (Sharpe and Cooper, 1998b). Although the ability of the inhibited fraction of CcO to oxidise cytochrome c is diminished – leading to an increased reduction of cytochromes cc_1 , this does not result in a decrease in respiration. In a process described as ‘branching’ (Chance et al., 1970), cytochrome c – an electron carrier with free movement in the inter-membrane space – can transfer an electron to a neighbouring NO-free CcO, thus continuing the movement of electrons along the chain towards O_2 . The accumulation of cytochrome c in its reduced form effectively increases the reductive pressure on the remaining NO-free CcO, hence, by the laws of mass action, increasing the flux of electrons through the uninhibited population, enabling respiration to be maintained. The fact that cytochrome b_H maintains its redox state (data not shown) (see also Hollis et al., 2003) while cytochromes cc_1 and aa_3 become more reduced suggests that the electron flux through the Q-cycle of complex bc_1 (complex III) remains constant and the bottleneck (i.e. the accumulation of electrons due to the partial inhibition of CcO by NO) occurs downstream of cytochrome b_H . However, the combined measurement of cytochromes c and c_1 from complex bc_1 (see Materials and Methods) does not allow us currently to determine whether electrons accumulate predominantly on c or c_1 .

As the concentration of NO increases, this compensatory process will clearly be exhausted owing to the finite limit of eTN in the remaining NO-free CcO. Therefore, when a critical fraction of the CcO population is inhibited, eTN in the NO-free CcO will reach a maximum, and further interaction with NO can only result in an inhibition of respiration. The existence of this critical fraction is indicated by the fact that the percentage change from the baseline in reduced cytochromes aa_3 at the O_2 concentration at which respiration becomes inhibited does not change significantly (from ~19%) with increasing L-arginine.

We have demonstrated that cells respiring at high steady-state eTN of CcO have less ability to maintain respiration after

treatment with the same concentration of L-arginine than cells respiring at low steady-state eTN. This can be explained by the fact that cells respiring at a higher eTN of CcO are closer to the limit of the excess capacity. Therefore, their ability to compensate for a progressive inhibition by NO, by increasing eTN in the NO-free enzyme population, is diminished. Furthermore, the nature of the interaction between NO and CcO depends on the redox species populating the catalytic centre of the CcO, which in turn depends on the eTN of CcO (Sarti et al., 2000; Giuffre et al., 2000; Mason et al., 2006). Simulations have shown that, under conditions of high $[O_2]$, low reductive pressure from cytochrome c and low eTN of CcO, the oxidised intermediate species of the catalytic centre are more populated than the reduced species, whereas at low $[O_2]$, high reductive pressure and high eTN the reduced species accumulate (Giuffre et al., 2000). NO interacts, in competition with O_2 , with the fully reduced species at a rate of $1 \times 10^8 \text{ M}^{-1} \text{ second}^{-1}$ (Blackmore et al., 1991). However, unlike O_2 , NO can also interact with Cu_B in the oxidised intermediates (Cooper et al., 1997; Torres et al., 1998; Giuffre et al., 1998), albeit at a much lower rate ($0.1 \times 10^5 \text{ M}^{-1} \text{ second}^{-1}$) than for the reaction with the reduced species (Giuffre et al., 2000). We may attribute the observed reduction of cytochromes aa_3 at an $[O_2]$ at which there is no inhibition of respiration to the interaction of NO with the oxidised species of the catalytic cycle that prevail at high $[O_2]$ and low eTN of CcO. As the high-affinity interaction of NO with the reduced species – accumulating as a consequence of the decreasing $[O_2]$ and increasing eTN – becomes more significant, respiration is inhibited. This explains the apparent decrease in the ability of cells to maintain respiration when working at a higher steady-state eTN of CcO, because accumulation of the reduced species, and hence inhibition of respiration, will occur at a higher $[O_2]$. The current VLS system does not allow us to determine spectroscopically the $[O_2]$ at which the population of redox species in the catalytic centre of CcO changes from predominantly oxidised to predominantly reduced. Experiments to study redox changes in cytochrome a_3 alone, coupled with a better understanding of the fate of NO at different $[O_2]$, are therefore underway.

Our hypothesis allows us to predict that cells with a low energy demand will respire with a low eTN of CcO; therefore, the catalytic centre will be populated with a higher proportion of oxidised species. Cells of tissues with a high demand for ATP will respire with a higher eTN of CcO, which will therefore be populated with a relatively higher proportion of reduced species. In this situation CcO will bind more NO inside the catalytic centre, but the enzyme will have less capacity to maintain respiration if the concentration of NO increases or O_2 decreases. It remains to be elucidated how biological situations in which modifications occur to the three factors involved in these interactions, namely [NO], $[O_2]$ and eTN, affect the internal milieu of cell and subsequent signalling.

Materials and Methods

Reagents

Sodium ascorbate, L-arginine, sodium dithionite, S-ethylisothiouria (S-EITU), myxothiazol, sodium nitrite, soybean protease inhibitor, tetracycline and N,N,N',N' tetramethyl-p-phenylenediamine (TMPD) were purchased from Aldrich-Sigma. Cell culture media, hygromycin and trypsin-EDTA were from Invitrogen. Blasticidin was from Calbiochem.

Plasmid preparation and transfection

The tetracycline-inducible cell line Tet-iNOS 293 that stably expresses NOS from human chondrocytes was obtained as described previously (Mateo et al., 2003). Briefly, the cDNA encoding the complete coding region of the NOS gene (GenBank accession no. X73029) was cloned into the inducible expression vector pcDNA5/FRT/TO (Invitrogen) using PCR primers designed to contain restriction sites for *HindIII* and *XhoI* at the 5' and 3' ends, respectively, giving as a result the pcDNA5/FRT/TO-iNOS DNA construct. For transfection, 2×10^6 Flp-In™ T-REx™-293 cells (Invitrogen), which stably express the tetracycline repressor, were co-transfected with 0.3 μg of pcDNA5/FRT/TO-iNOS and 3 μg of Flp recombinase expression plasmid (pOG44) using 7.5 μl of lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Forty-eight hours after transfection, the cells were selected in growth medium supplemented with 200 $\mu\text{g ml}^{-1}$ hygromycin B and 15 $\mu\text{g ml}^{-1}$ blasticidin.

Cell culture and induction of human NOS in Tet-iNOS293 cells

Tet-iNOS 293 cells were cultured in T-175 flasks with Phenol-Red-free Dulbecco's modified Eagle's medium (DMEM) containing 25 mM D-glucose, 4 mM glutamine, 15 $\mu\text{g ml}^{-1}$ blasticidin, 200 $\mu\text{g ml}^{-1}$ hygromycin B and 10% (v/v) of heat-inactivated foetal bovine serum (HI-FBS), as previously described (Mateo et al., 2003). Maximal expression of human NOS in Tet-iNOS293 cells was achieved by 15 hours incubation with induction medium (DMEM containing 25 mM D-glucose, 4 mM glutamine, 10% HI-FBS, 1.5 $\mu\text{g ml}^{-1}$ tetracycline and 500 μM S-EITU; the latter is added to avoid generation of NO from L-arginine in the medium that is necessary for NOS dimerisation and consequent enzyme activity). Under these conditions we achieved a sensitive and reproducible NO generation from NOS in response to small concentrations of exogenous L-arginine. A basal production of NO was evident in these cells (see Results) even in the absence of exogenous L-arginine, most probably due to the conversion of endogenously-generated L-arginine by NOS once the inhibitor (S-EITU) was removed.

Cells were harvested by trypsinisation, then centrifuged at 115 g for 10 minutes and re-suspended at a concentration of 5×10^6 cells ml^{-1} 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 HI-FBS). Cell viability was always over 95%, as measured by the Trypan Blue exclusion method. Cell suspensions were placed in a water bath at 37°C with constant agitation (80 rpm) to ensure that they remained well-oxygenated and at a constant temperature throughout the experiments. After 1 hour incubation to remove the NOS inhibitor, cells were centrifuged again and re-suspended at a cell concentration of approximately 2×10^7 cells ml^{-1} in Hanks-VLS solution. Cells were then incubated for another hour at 37°C in the water bath before placing them in the VLS chamber in aliquots of 1 ml final volume. At the end of each experiment aliquots of 50 μl were diluted in triplicate, in 10 ml of isotonic buffer, and cell-counting was immediately carried out using a Coulter Counter (Z Series, Beckman Coulter; FL). L-arginine was always added when the concentration of O_2 in the chamber reached (approximately) 70 μM . This $[O_2]$ was selected because both parameters of our study, respiration and the redox state of cytochromes aa_3 and cc_1 , remained independent of $[O_2]$ above $\sim 50 \mu\text{M}$ (in the absence of exogenous L-arginine, significant changes from the baselines occurred in cytochromes aa_3 , and respiration at 34.4 ± 1.1 and $9.8 \pm 1.1 \mu\text{M } O_2$, respectively).

Simultaneous measurement of cytochrome redox states, respiration and NO release from exogenous L-arginine

The visible-light spectroscopy (VLS) system is essentially the same as described before (Hollis et al., 2003) with some improvements in the optical system and the detection of NO. Briefly, the diffraction grating has been replaced with one blazed at 400 nm (Horiba-Jobin Yvon, Stanmore, UK), for greater sensitivity of light detection in the visible region, and the optical fibres with those of a larger core diameter and numerical aperture (Thorlabs, Ely, UK), for enhanced delivery and collection of light. These improvements to the system allow the sampling rate to be increased from 50 to 100 Hz, although averaging is maintained such that a data point or spectrum is recorded every 500 microseconds. The NO electrode has been replaced by a nanosensor (amiNO-700; Innovative Instruments, FL), calibrated using standard concentrations of NaNO_2 in the reaction with 10 mM KI and 100 mM H_2SO_4 at 37°C. The sensitivity of the electrode to the generation of NO was on average over 250 pA nM^{-1} . Calibration of the Clarke-type O_2 electrode (Rank Bros., Cambridge, UK) and corrections for its time response and background O_2 consumption were carried out as previously described (Hollis et al., 2003). The rate of mitochondrial respiration (VO_2) was determined as the time derivative of the $[O_2]$.

Measured changes in optical attenuation are converted into changes in the redox states of the mitochondrial cytochromes by two multi-wavelength linear least-squares fits of their specific absorption coefficients (Hollis et al., 2003). To account for non-absorption changes in the attenuation spectra, a first-order background has been included in the least-squares fitting algorithm, described by $(x-\lambda)+y$, where λ is the wavelength and x and y are the free (variable) coefficients of the first-order background. Simulations have been carried out to demonstrate the ability of the fitting to recover redox-dependent changes in cytochrome concentrations within the range of the redox changes observed here (data not shown).

The measurement of pathlength, required for the absolute determination of redox-dependent changes in cytochrome concentrations, was carried out as previously described (Hollis et al., 2003). Within the range of cell concentrations used here, a significant ($P < 0.001$) negative correlation between cell concentration and pathlength was observed. Thus, the pathlength (β) was calculated from the cell concentration using the equation $\beta = p \cdot [\text{cell}] + q$, where $[\text{cell}]$ is the cell concentration and the coefficients p and q were $-0.23 \pm 0.01 \text{ cm } 10^{-7} \text{ cells ml}^{-1}$ and $2.56 \pm 0.03 \text{ cm}$, respectively ($n=16$).

Quantification of changes in respiration rate and reduction of cytochromes aa₃ and cc₁

Changes in the redox states of cytochromes aa₃ and cc₁ are expressed as percentage changes varying between 0% at the [O₂]-independent baseline (prior to addition of L-arginine at $\sim 70 \mu\text{M O}_2$) and 100% when fully reduced at anoxia. It should be noted, however, that the redox states of cytochromes aa₃ and cc₁ at the [O₂]-independent baseline are not 0%, i.e. fully oxidised. This assignment was designed to provide a better comparison with VO₂, for which the maximal value during the steady-state was normalised to 100%. Using the method described below to determine total cytochrome concentrations, the percentage of cytochromes aa₃ and cc₁ in their reduced forms at the [O₂]-independent baseline was estimated to be 11.8 ± 1.4 and $11.4 \pm 1.6\%$, respectively ($n=6$). For the case of cytochromes aa₃ this predominantly (80–90%) reflects the redox state of cytochrome a and is not used to draw conclusions about the redox species populating the catalytic centre (a₃-Cu_B). The measurement of cytochromes cc₁ comprise a combined signal from cytochrome c₁ of complex bc₁ and cytochrome c in the intermembrane space, although the ratio of c:c₁ in Tet-iNOS293 cells is undetermined.

Estimation of total concentration of CcO and turnover number (TN) of the enzyme in vivo

The total CcO concentration ($[\text{CcO}]_{\text{total}}$) was estimated in respiring cells by measuring from the baseline the maximal reduction of cytochrome aa₃ at anoxia ($\Delta[\text{aa}_3]_{\text{red}}$) and, in an independent experiment, the maximal oxidation obtained after the addition of $1 \mu\text{M}$ of myxothiazol ($\Delta[\text{aa}_3]_{\text{oxi}}$), i.e. $[\text{CcO}]_{\text{total}} = (\Delta[\text{aa}_3]_{\text{red}} + \Delta[\text{aa}_3]_{\text{oxi}}) / \beta$, where β is the pathlength determined from cell number as described above. Electron turnover (eTN) of the enzyme (in electrons per second) was then estimated from the total concentration of the enzyme and the maximal VO₂ during the [O₂]-independent phase (VO_{2max}) using the equation $e\text{TN} = \text{VO}_{2\text{max}} \cdot 4 / [\text{CcO}]_{\text{total}}$, the factor 4 accounting for the number of electrons in one turnover cycle of CcO, i.e. the consumption of one molecule of O₂.

Statistical analysis

The mean \pm standard deviation was determined for the quantitative analysis of the results. The (two-tailed) Z-test was used to determine statistically significant changes in VO₂ and cytochromes aa₃ and cc₁ from their [O₂]-independent baseline values, and the (one-tailed) *t*-test was used to determine statistically significant correlations between dependent and independent variables (pathlength vs cell concentration and eTN vs decrease in respiration).

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