Control of Mitochondrial Respiration by NO', Effects of Low Oxygen and Respiratory State*

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Nitric oxide (NO') inhibits mitochondrial respiration by binding to the binuclear heme a\textsubscript{3}Cu\textsubscript{b} center in cytochrome c oxidase. However, the significance of this reaction at physiological O\textsubscript{2} levels (5–10 \textmu M) and the effects of respiratory state are unknown. In this study mitochondrial respiration, absorption spectra, [O\textsubscript{2}], and [NO'] were measured simultaneously at physiological O\textsubscript{2} levels with constant O\textsubscript{2} delivery, to model in vivo respiratory dynamics. Under these conditions NO' inhibited mitochondrial respiration with an IC\textsubscript{50} of 0.14 ± 0.01 \textmu M in state 3 versus 0.31 ± 0.04 \textmu M in state 4. Spectral data indicate that the higher sensitivity of state 3 respiration to NO' is due to greater control over respiration by an NO'-dependent spectral species in the respiratory chain in this state. These results are discussed in the context of regulation of respiration by NO' in vivo and its implications for the control of vessel-parenchymal O\textsubscript{2} gradients.

The interaction of nitric oxide (NO') with mitochondria is now emerging as one of the major pathways through which NO' can exert both physiological and pathological responses in a variety of cell types (1, 2). The most sensitive and widely studied target for NO' in mitochondria is the terminal enzyme of the electron transport chain, cytochrome c oxidase (3–6). The binuclear O\textsubscript{2} binding site of this enzyme (heme a\textsubscript{3}Cu\textsubscript{b}) is reversibly inhibited by NO' (3, 7, 8). This is controlled by O\textsubscript{2} at two levels: by a competition between NO' and O\textsubscript{2} at the enzyme's active site (9), and by the partition of both NO' and O\textsubscript{2} into mitochondrial membranes, which accelerates the direct reaction between these molecules (10, 11).

Despite an acknowledged physiological existence of the NO'-cytochrome c oxidase interaction, its biological importance and function remain uncertain. Three of the likely physiological roles for this interaction are: (a) regulation of mitochondrial H\textsubscript{2}O\textsubscript{2} generation (2, 12), (b) regulation of cytochrome c release in apoptosis (13), and (c) prevention of hypoxia in respiring tissues by inhibiting mitochondrial respiration and extending O\textsubscript{2} diffusion gradients from blood vessels (14). In pathological situations, perturbation of the NO'-cytochrome c oxidase pathway can occur, resulting in enhanced apoptosis or loss of control over respiration, as has been demonstrated in several tissues (15–17).

Critical to understanding the mechanisms regulating the NO'-cytochrome c oxidase pathway are fundamental biochemical questions that have not been addressed in detail. For example, different NO'-derived species bound to purified cytochrome c oxidase have been identified, including a putative heme a\textsubscript{3}Cu\textsubscript{b}-nitrite complex (7), but it is unknown which of these complexes is formed in respiring mitochondria. In addition, the sensitivity of mitochondrial respiration to NO' appears greater while actively respiring (state 3 in the isolated preparation of the organelle) than while quiescent (state 4) (18). This phenomenon represents an additional level of control over respiration. However, it is inherently linked to the regulation of tissue O\textsubscript{2} levels, because mitochondrial O\textsubscript{2} consumption is different in each respiratory state. Therefore it is essential to determine the control of state 3 and 4 respiration by NO' under conditions of O\textsubscript{2} delivery similar to those in vivo.

Mechanisms that could explain the greater sensitivity of state 3 respiration to NO' include: (a) the formation of different NO'-derived complexes in cytochrome c oxidase depending on electron flux (7), (b) different amounts of such complexes formed in each state, or (c) different levels of control over respiration exhibited by cytochrome c oxidase in each state (18, 19). The concept of control (i.e. flux control coefficient (19–22)) has received little attention in the context of NO' regulation of respiration. This is important to study because control is a changing parameter, with cytochrome c oxidase exhibiting more control over respiration in state 3 than in state 4 (19). In cells, it is likely that mitochondria can exist in a mixture of both respiratory states.

In this investigation, several of the above issues were addressed by measuring the effects of NO' on mitochondrial respiration under conditions of controlled O\textsubscript{2} delivery (23). In addition mitochondrial absorption spectra were acquired to provide information on the redox status of cytochromes and their NO'-derived complexes. The results indicate that mitochondrial state 3 respiration is indeed more sensitive to NO'-dependent inhibition than state 4 under controlled O\textsubscript{2}-delivery conditions. Analysis of the absorption spectra suggested a common NO'-dependent spectral intermediate is formed to a simi-
lar extent in both respiratory states. Interestingly, the data suggest that the inhibition of respiration due to population of this species is greater in state 3. In addition, NO-dependent inhibition of respiration elevated the local [O$_2$], as has been proposed to occur in vivo (14). The implications of these findings are discussed in the context of a role for NO as a physiological regulator of both mitochondrial respiration and local O$_2$ gradients.

**MATERIALS AND METHODS**

**Chemicals and Animals**—Sucrose for mitochondrial preparations was ultra-pure grade from USB (Cleveland, OH). All other chemicals were from Sigma. Male Sprague-Dawley rats, 250 g in weight, were purchased from Harlan and housed according to standard humane procedures on a 12 h light/dark cycle with food and water available ad libitum. Pure solutions of NO$^*$ gas were prepared by bubbling NaOH-scrubbed commercial NO$^*$ gas through deoxygenated (argon-purged) water in a gas-tight bulb (24). Solutions were sealed under NO$^*$ atmosphere, stored in the dark, and aliquots withdrawn through a gas-tight septum into a gas-tight syringe. The concentration of NO$^*$ in these stock solutions was determined spectrophotometrically by monitoring oxyhemoglobin to methemoglobin conversion and was typically ~1.5 mM. The NO probe and NO$^*$ solutions were also calibrated using an acidified nitrite/potassium iodide method as per the manufacturer’s instructions (WPI Inc., Sarasota, FL).

**Isolation of Mitochondria**—Rat liver mitochondria were isolated by standard differential centrifugation techniques in buffer comprising sucrose (250 mM), Tris (10 mM), and EGTA (1 mM), pH 7.4 (24). All steps were performed at 4 $^\circ$C, and mitochondria were stored on ice and used within 4 h of isolation. Mitochondria in this study had respiratory control ratio values of 4.7 ± 0.1 with glutamate + malate as substrates. Protein was determined using the Folin-phenol reagent against a standard curve constructed using bovine serum albumin (25).

**Procedures**—Five ml of respiration buffer comprising KCl (120 mM), sucrose (25 mM), HEPES (10 mM), EGTA (1 mM), KH$_2$PO$_4$ (1 mM), MgCl$_2$ (5 mM), glutamate (15 mM), and malate (7.5 mM), pH 7.3, was added to the sample cuvette. The apparatus was assembled and purged with 100% N$_2$ to lower the liquid phase [O$_2$] prior to mitochondrial addition. After addition of mitochondria (1 mg protein/ml), the gas flow mixture was re-adjusted to give a steady-state [O$_2$] of ~5 µM. For state 4 respiration (with glutamate plus malate alone, formally state 2), the mixture required 8% air, whereas state 3 respiration (with the additional presence of 2 mM ADP) required 39% air. In all cases, substrate and/or ADP concentrations were sufficient to maintain steady-state respiration for >55 min.

The range of cellular O$_2$ concentrations that are present in vivo have been investigated extensively, with precise values dependent upon the specific tissue, metabolic activity, and subcellular location. In this investigation, an initial value of ~5 µM was chosen, being at the low range of values experienced by mitochondria (26, 27).

After a steady-state O$_2$ concentration had been maintained for at least 3 min (indicating O$_2$ consumption = O$_2$ delivery), NO$^*$ was added in the form of a bolus injection (1 µM final), and the kinetics of solution [O$_4$] and [NO$^*$] were continuously monitored. Absorption spectra (500–400 nm, 1 nm resolution) were acquired at ~75 s intervals during the NO$^*$ addition, until [O$_4$] returned to the same steady-state level as before NO$^*$ addition, indicating recovery of mitochondrial respiration. In the case of NO$^*$ it is technically difficult to establish a steady-state concentration of this inhibitor within the time-frame of this experiment, and thus it is necessary to capture data in the non-steady-state.

Mass transfer coefficients (see Equation 1) were also determined for each experiment. In a series of experiments, absorption spectra were acquired in the range 380–640 nm, under half-saturating O$_2$ (~95 µM), for mitochondria respiring in state 3, in the presence of either antimycin A, cyanide, or NO$^*$.

**Calculation of Mitochondrial O$_2$ Consumption Rates**—The underlying principle used to calculate steady-state O$_2$ consumption rates in open flow respirometry is that the O$_2$ consumed is proportional to the predicted liquid phase [O$_2$], minus the measured liquid phase [O$_2$]. Cole et al. (23) expressed this algebraically as follows (Equation 1),

$$Q = m(C^*-C_1)$$  \hspace{1cm} \text{(Eq. 1)}$$

where $Q$ is mitochondrial respiration rate, $C^*$ is the predicted liquid phase [O$_2$], based on gas phase pO$_2$ multiplied by the O$_2$ solubility in the liquid phase (see next paragraph), $C_1$ is the liquid phase [O$_2$] determined from the O$_2$ sensor, and $m$ is the mass transfer coefficient (23). The term $m$ is a kinetic constant defining the first order response of the liquid phase [O$_2$] to a step change in gas phase [O$_2$]. Experimentally, $m$ is determined by recording the decay of liquid phase [O$_2$] in response to switching the gas phase from 100 ml/min of air to 100 ml/min of pure N$_2$. Typical values of $m$ in this system were 0.0035 ± 0.0002 s$^{-1}$.

Average atmospheric pressure during the course of these experiments was 98.5 kPa. The concentration of O$_2$ in air-saturated mitochondrial respiration buffer at 37 $^\circ$C (C$^*$) was thus calculated as 196.8 µM (26). Throughout this paper [O$_2$] is referred to in µM, calculated from the mV output of the polarographic O$_2$ sensor, because this is the term most commonly used in mitochondrial physiology. Fig. 1 shows a typical set of traces for a mitochondrial respiration experiment. Following addition of mitochondria and respiratory substrates, a new steady-state value of O$_2$ is established. Under these conditions, the rate of O$_2$ consumption (Q) is determined by Equation 1.

Following addition of NO$^*$, transient inhibition of respiration causes a rise in $C_1$, and because the system is now no longer at steady-state Equation 1 does not apply. Thus, a new equation for the calculation of rate (Q) is required. From general engineering principles, the equation for the rate of change of an intermediate X (i.e. dX/dt) under all condi-
tions (both steady- and non-steady-state) is as follows,

$$\frac{dX}{dt} = A - B \quad (\text{Eq. 2})$$

where A is the rate of delivery of X, and B is the rate of consumption of X. In the current experimental system, A equates to $O_2$ delivery, i.e. $m(C^* - C_1)$, and B equates to mitochondrial $O_2$ consumption rate ($Q$). The intermediate X is the liquid phase $O_2$ concentration ($C_1$). Thus, substituting these terms into Equation 2 yields the following,

$$\frac{dC_1}{dt} = m(C^* - C_1) - Q \quad (\text{Eq. 3})$$

and subsequent rearrangement of this equation yields a universal equation for the calculation of $Q$ under all conditions (both steady- and non-steady-state).

$$Q = m(C^* - C_1) - \frac{dC_1}{dt} \quad (\text{Eq. 4})$$

Experimentally, $dC_1/dt$ is the slope of the measured liquid phase $[O_2]$ trace ($C_1$). Examining Equation 4, it becomes clear that in the steady-state ($dC_1/dt = 0$), it simplifies to Equation 1. In fact, rather than considering Equation 4 as a derivative of Equation 1, it is somewhat beneficial to consider Equation 1 and the steady-state, as a subset of the conditions governed by the universal Equation 4.

In adapting these terms for application to the non-steady-state, the rate-limiting step in obtaining a value for $C_1$ is the response time of the liquid phase $[O_2]$ to changes in $Q$, and this is governed by $m$. Neither the time constant of the $O_2$ sensor (6.5 ± 0.1 s, n = 6), nor the adjustment of mitochondria to a new respiration rate (~100 ms (29)) are limiting factors. Supporting this, it was observed that $C_1$ changed almost instantaneously upon addition of NO to respiring mitochondria (Fig. 1). Thus, the term $dC_1/dt$ accurately describes the rate of change of liquid phase $[O_2]$ in response to changes in mitochondrial respiration rate in the non-steady-state condition. Herein, rates are primarily expressed as a percentage of the rate prior to NO$^+$ addition.

Correction of Rates for Limitation at Low $[O_2]$—During initial experiments, it was observed that mitochondrial respiration was $O_2$-limited at the values of baseline $[O_2]$ chosen (~5 μM). Addition of NO$^+$ and subsequent inhibition of respiration then caused a transient increase in $C_1$, which could possibly give rise to variable $O_2$ limitation throughout the course of the experiment. It was therefore decided to account for this phenomenon by determining the steady-state $O_2$ consumption rate ($Q$) as a function of $[O_2]$ for this experimental system. A series of incubations were performed as described under “Experimental Procedures,” without NO$^+$ at different values of $C^*$, allowing the system to reach steady-state. The rate $Q$ was calculated using Equation 1. This data set was then used to correct the experimental data by multiplying each point on the absolute rate plots by a correction factor specific to the $[O_2]$ at that point. In practice, this was achieved by determining the $[O_2]$ for each point on Fig. 3A (by reference to the original data, Fig. 2B). Then, from Fig. 4A, the percentage rate was read from the y-axis at that particular value of $[O_2]$ and converted to a fractional value (50% = 0.5, 60% = 0.6 etc.).

A representative trace for $[O_2]$ and $[NO_3]$ is shown in Fig. 2. Upon addition of NO$^+$ to returning the value to a normalized rate (120 μM).

**RESULTS**

This study represents the first use of an open-flow respirometry system to determine mitochondrial respiration rates under non-steady-state conditions. In the first series of experiments (Fig. 2) both $O_2$ and NO$^+$ were monitored simultaneously in respiring mitochondria in states 3 and 4 (thick line and thin lines, respectively). Liquid phase oxygen ($C_1$) was maintained at ~5 μM prior to addition of a 1 μM bolus of NO$^+$ (shown by the arrow), which then caused an increase in $C_1$ in both states. Following the decay of NO$^+$, $C_1$ returned to a similar level as that seen before NO$^+$ addition, consistent with the reversal of respiratory inhibition. The increase in $[O_2]$ upon addition of NO$^+$ was more rapid and reached a higher level in state 3 than in state 4, primarily because the proportion of air in the gas mixture used to support state 3 (39%) is greater than for state 4 (8%). This means that, for example, even if $Q$ were identical for both states after NO$^+$ addition, the dissolved $O_2$ concentration will rise more rapidly for state 3 because the gas phase $pO_2$ is nearly 5-fold higher. The kinetics of NO$^+$ decay (Fig. 2, C and D) were not significantly different between respiratory states.

From a series of identical experiments, mitochondrial respiration rates ($Q$) were calculated and are shown in Fig. 3, both as a function of the time from NO$^+$ addition (panels A and B) and the corresponding NO$^+$ concentrations (panel C). The IC$_50$ for NO$^+$ inhibition occurs at 0.24 ± 0.03 μM in state 3 and 0.39 ± 0.04 μM in state 4. In addition to the absolute values of $Q$ (nmols $O_2$/min/mg protein, panel A), rates are also expressed as a percentage of the initial (pre-NO$^+$) rate (panel B).

Interestingly, respiration rates increased to a value greater than the initial rate prior to addition of NO$^+$ at the later time points. We attribute this response to $O_2$-limitation of mitochondrial respiration at low $[O_2]$. Upon addition of NO$^+$, the inhibition of respiration results in an increase in $[O_2]$ (see Fig. 2, A and B). At later time points when most of the NO$^+$ has decayed, $[O_2]$ is still higher than the initial level of 5 μM. Thus the observed rate of respiration is greater than the value seen prior to NO$^+$ addition. Eventually, as $[O_2]$ returns to baseline levels, the rate recovers to the initial value, because $O_2$ is once again limiting.

The increase in $Q$ above 100% is greater here than in state 4 (see Fig. 2), both $O_2$ and NO$^+$, respectively, as a function of time, under conditions of state 3 (thick line) and state 4 (thin line) respiration. $B$ and $D$, show averages for 4 such experiments ± S.E. Filled symbols, state 3; open symbols, state 4. NO$^+$ was added (bolus addition, 1 μM final concentration) at the time indicated by the dashed arrow.
These data are shown in Fig. 4A and demonstrate that mitochondrial respiration is O\textsubscript{2} limited in this experimental system at \(<20\, \mu\text{M} \text{O}_2\) (50 ± 2.5 \(\mu\text{M}\)).

Absorption spectra were also acquired in this experiment, and difference spectra were obtained by subtracting the spectrum at 120 \(\mu\text{M} \text{O}_2\) from those obtained at lower values of [O\textsubscript{2}]. The absorbance for the peak at 445 nm was then expressed as a percentage of the fully reduced spectrum obtained in the presence of sodium dithionite (Fig. 4B). This represents the fractional reduction of cytochromes aa\textsubscript{3} in cytochrome c oxidase. Consistent with inhibition of mitochondrial respiration at low [O\textsubscript{2}], these data indicate that the respiratory chain is more reduced as [O\textsubscript{2}] falls. This is in contrast to a previous criticism of open-flow respirometry, in which a mis-match between respiration rates and cytochrome redox status was included as a factor in calculating rates, mitochondrial respiration is O\textsubscript{2} limited in this experimental system at \(<20\, \mu\text{M} \text{O}_2\) (50 ± 2.5 \(\mu\text{M}\)).

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whereas addition of CN\textsuperscript{−} results in the appearance of a peak at 550 nm, corresponding to reduction of cytochrome c due to downstream inhibition of complex IV. Antimycin A also gives a peak at 432 nm, which can be attributed to reduction of the b cytochromes. In the case of CN\textsuperscript{−}, the resultant peaks at 445 and 602 nm correspond to a combination of reduced cytochromes aa\textsubscript{3} and the a\textsubscript{3}-CN derivative. The peak at 424 nm is likely due to a combination of reduced cytochromes b, c, and c\textsubscript{1}.

With NO\textsuperscript{−}, a broad, high amplitude peak at ~431 nm is present. This peak is consistent with the formation of the a\textsubscript{3}-NO\textsuperscript{−} complex at 430 nm (7), with a likely contribution from other reduced upstream cytochromes. However, any contribution from the b cytochromes is likely to be small under these conditions, because the maximum additional reduction of the b cytochromes attainable with antimycin A (see above) is significantly less than the peak at 431 nm with NO\textsuperscript{−}. The remainder of the absorbance at 431 nm with NO\textsuperscript{−} is likely due to a composite of reduced cytochromes c, c\textsubscript{1}, and aa\textsubscript{3}. However, the reduced aa\textsubscript{3} peak at 445 nm may be lost in the shoulder of the 432 nm peak, especially because the a\textsubscript{3}-NO\textsuperscript{−} complex is blue-shifted relative to aa\textsubscript{3}. In particular, NO\textsuperscript{−} exposure results in a peak at 550 nm, attributable to reduced cytochrome c, and thus it is likely that at least part of the Soret peak with NO\textsuperscript{−} is due to reduction of this cytochrome as an indirect consequence of inhibition of complex IV.

By examining the data in Fig. 6 alongside those in Fig. 5, it is possible to gain further insight into the differences between NO\textsuperscript{−} inhibition of state 3 and state 4 respiration. The magnitude of inhibition of state 4 respiration increases much more rapidly than that of state 3 respiration, which is consistent with the idea that inhibition of complex IV is the primary cause of inhibition of state 4 respiration. The results also suggest that NO\textsuperscript{−} inhibition of state 3 respiration is due to inhibition of both complexes III and IV.

Fig. 5. NO\textsuperscript{−} inhibition of state 3 and state 4 respiration, correcting for O\textsubscript{2} limitation. Data from Fig. 3 were re-scaled according to the O\textsubscript{2} limitation data shown in Fig. 4A (see “Materials and Methods”). Briefly, for each data point the value of [O\textsubscript{2}]/(O\textsubscript{2}) was measured, and the corresponding degree of O\textsubscript{2} limitation was extrapolated from Fig. 4A and as a correction factor to calculate a new value for Q. A, rates expressed in absolute terms (O\textsubscript{2} corrected Q, nmols O\textsubscript{2}/min/mg protein) as a function of time. B, rates expressed as a percentage of the initial, pre-NO\textsuperscript{−} rate, as a function of time. Data obtained from A, C, rates expressed as a percentage of the initial, pre-NO\textsuperscript{−} rate, as a function of [NO\textsuperscript{−}]. Filled symbols, state 3; open symbols, state 4. Means ± S.E., n = 4. Asterisks indicate statistical significance (p < 0.05) between state 3 and state 4 data points.

Fig. 6. Absorption spectra of NO\textsuperscript{−}-inhibited mitochondria in state 3 versus state 4. Panels on the left show absolute absorbance spectra were taken at various time points during NO\textsuperscript{−} inhibition in state 3 (A) and state 4 (B). Thick lines show the initial baseline spectra (labeled Pre-NO\textsuperscript{−}) and fully inhibited spectra (labeled Max [NO\textsuperscript{−}]). Thin lines show intermediate spectra. The dotted lines (labeled End) show the final spectra after full decay of NO\textsuperscript{−} and recovery of respiration rates. Panels on the right show difference spectra calculated by subtracting the baseline spectrum in each case (i.e., without NO\textsuperscript{−}) from those acquired during NO\textsuperscript{−} exposure. The dotted arrows on difference spectra indicate the direction of movement with increasing NO\textsuperscript{−} concentration. Data are representative of 4 independent experiments.

Fig. 7. Mitochondrial absorption spectra with various inhibitors. To mitochondria respiring in state 3 at half-saturating [O\textsubscript{2}] (~95 \textmu M) was added either antimycin A (1 \textmu M), KCN (1 \textmu M), or NO\textsuperscript{−} (3 \textmu M). Spectra were acquired both before addition of inhibitors and 30 s afterward. Data shown are averaged difference spectra (n = 2), i.e., subtracting the spectrum in the absence of inhibitor, from the spectrum in the presence of inhibitor. Resolution = 0.5 nm.
The primary findings of this study are: (i) mitochondrial respiration is more sensitive to NO⁻ in state 3 than in state 4 at physiological O₂ levels, (ii) open flow respirometry (23) can be applied to examine mitochondrial function under non-steady-state conditions, (iii) mitochondrial respiration may be O₂-limited at higher concentrations than previously thought, and (iv) inhibition of respiration by NO⁻ is completely reversible in both state 3 and state 4. Additionally, mitochondrial respiratory inhibition by NO⁻ appears to elevate local [O₂] in this constant O₂-delivery system. In vivo, such a response may facilitate enhanced vessel-parenchymal O₂ diffusion, as previously proposed (14). Furthermore, the differential sensitivity of states 3 and 4 to NO⁻ suggests that O₂ would be delivered to distances further from a vessel under conditions of increased work demand. This is because mitochondria in working tissues are in a state more akin to state 3, and thus more sensitive to NO⁻ inhibition, as opposed to quiescent tissues, which would be in state 4 and thus less sensitive to NO⁻.

Because NO⁻-dependent inhibition of respiration is competitive with O₂, previous reports (18) showing greater sensitivity of state 3 respiration to NO⁻ were biased by the fact that these observations were made over a range of lower O₂ concentrations in state 3 than in state 4. In contrast, the open-flow respirometer yields more limited changes in [O₂] that are actually greater in state 3 (see Fig. 2), and thus competition with O₂ cannot account for the greater NO⁻ sensitivity of state 3 respiration reported here.

Having confirmed that under physiological conditions, state 3 respiration is indeed more sensitive to NO⁻ than is state 4, we sought to investigate the mechanisms involved. Fig. 6 suggests that the difference is not due to formation of a distinct NO⁻-derived species in each respiratory state, whereas Fig. 7 suggests that the amount of the common NO⁻-dependent spectral species formed is not different in each state.

Although the precise identity of the NO⁻-dependent spectral species is not known, it is likely a composite of both the heme a₃-NO⁻ complex and the upstream reduced cytochromes b, c, and c₁. This assignment is based on the difference spectra acquired with respiratory inhibitors shown in Fig. 7. Unfortunately, acquisition of spectra over such a wide range of wavelengths (380–640 nm at 0.5 nm resolution) takes ~2.5 min in this system, and this was incompatible with the rapidly changing kinetics of both [NO⁻], [O₂], and mitochondrial respiration. Thus, a smaller window of wavelengths (400–500 nm, 1 nm resolution) was examined in the NO⁻ kinetic experiments, to maximize the time-resolution of this study. However, in doing so, it is acknowledged that some spectral information is lost. Thus, although high resolution kinetic studies on purified cytochrome c oxidase have reported the formation of a cytochrome a₃-nitrite complex within the enzyme, in addition to the a₃-NO⁻ complex (7), the lower relative resolution of the current spectral system does not allow us to address this point. However, our data does not preclude the possibility that in mitochondria and cells, different NO⁻-derived species could be formed at cytochrome c oxidase in different metabolic states.

Because the NO⁻-dependent spectral species in Fig. 6 appears to originate from an inhibited form of cytochrome c oxidase (see Figs. 6 and 7 and descriptions), it is possible to use these data to construct a control/threshold curve (20–22). At this stage it is important to define what is meant by control in this context. In an integrated system such as mitochondrial respiration, the component steps have varying degrees of control over the respiration rate. These include but are not limited to: the tricarboxylic acid cycle, the respiratory chain, proton leak, the phosphorylation machinery (ATP synthase, adenine-nucleotide translocase), and the various mitochondrial transmembrane solute carriers (19, 31). Control can be examined by determining the response of the overall system (respiration) to...
the inhibition of one of these components. The greater the degree of control by a given component, the greater will be the effect of inhibiting it on respiration. The term threshold refers to a characteristic elbow-shape in control-analysis curves (see Refs. 21 and 22) and is typically used to define the excess capacity of individual components of a system. A component with a larger threshold is present in greater excess, and thus inhibiting it has relatively little effect on respiration. Generally, a larger threshold equates to a lower degree of control.

Plotting the population of the NO-dependent spectral species against the respiration rate (Fig. 8B) reveals that this species has more control over respiration in state 3 than in state 4. These data are in agreement with previous control analysis studies (19, 31) of mitochondria, which have shown that in state 4 much of the control over respiration lies outside the respiratory chain (e.g. in the proton leak of the inner membrane), whereas in state 3 it lies mostly within the respiratory chain (in particular at cytochrome c oxidase). We therefore conclude that the predominant reason why respiration is more sensitive to NO in state 3 is due to a greater control over respiration by cytochrome c oxidase in this state.

Another important observation from the current data set is that mitochondrial respiration always returns to 100% of its initial rate following NO decay. This property has been difficult to determine previously because in closed-chamber experiments the full recovery of respiration occurs at low [O2], where the potency of NO is increased, resulting in a progressive inhibition of the respiration rate near the end of the trace (see Ref. 10 for an example). The present study conclusively demonstrates that NO is a fully reversible inhibitor of mitochondrial respiration.

With regard to the O2-sensitivity of mitochondrial respiration in this system (Fig. 4), the [O2] for half-maximal saturation of respiration rate (p50) was −2.5 μM. This is ~10× higher than previously published values for isolated mitochondria (26). However, several differences are present between this and previous experimental systems, including the use of an open-flow versus a closed respirometer chamber, the temperature, the concentration and source of mitochondria, and the dimensions of the open-flow system. The wide range of values for O2 p50 available in the literature (0.05–0.8 μM O2), as well as the current data, highlight the importance of determining p50 for each experimental system because no single value appears applicable to all conditions. Whether O2 limitation of mitochondrial respiration occurs in vivo over this range of [O2] (1–20 μM) remains to be determined, although the implications for such a limitation are profound.

Using the information on O2-limitation contained in Fig. 4, a transformation was performed on the respiration rate data in Fig. 3, to give O2-corrected respiration rates, shown in Fig. 5. Although it is recognized that these data (Fig. 5) are not true values of Q because they do not originate from equation 1, nevertheless the outcome of this analysis is still the same, i.e. that respiration is more sensitive to inhibition by NO in state 3 than in state 4.

The results of this paper, and in particular the rise in [O2] following NO addition to the chamber (Figs. 1 and 2), give further insight into the hypothesis that NO-dependent inhibition of mitochondrial O2 consumption, in the cells lining blood vessels, would allow O2 to diffuse further from the vessels and facilitate parenchymal cell respiration (14). These experiments support this general principle, although it is recognized that in vivo, O2 delivery is a complex variable that may itself respond to changes in respiration. In addition, other physiologic or pathologic functions could influence these responses including NO regulation of O2 gradients in O2-sensing tissues such as the carotid body.

In summary we have demonstrated that NO-dependent inhibition of mitochondrial respiration is responsive to both respiratory state and O2 tension. It is evident that NO has an important role in the cell as an endogenous physiological regulator of both mitochondrial respiration and of potential mitochondrial linked cell signaling events (2).

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