Nitric oxide regulation of mitochondrial oxygen consumption II: molecular mechanism and tissue physiology

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Cooper CE, Giulivi C. Nitric oxide regulation of mitochondrial oxygen consumption II: molecular mechanism and tissue physiology. Am J Physiol Cell Physiol 292: C1993–C2003, 2007. First published February 28, 2007; doi:10.1152/ajpcell.00310.2006.—Nitric oxide (NO) is known as an intercellular messenger, synthesized in mammalian systems from arginine, NADPH, and oxygen by the NO synthase (NOS) class of enzymes (1). It is an intercellular signaling molecule; among its many and varied roles are the control of blood flow and blood pressure via activation of the heme enzyme, soluble guanylate cyclase. A growing body of evidence suggests that an additional target for NO is the mitochondrial oxygen-consuming heme/copper enzyme, cytochrome c oxidase. This review describes the molecular mechanism of this interaction and the consequences for its likely physiological role. The oxygen reactive site in cytochrome oxidase contains both heme iron (a3) and copper (Cu) centers. NO inhibits cytochrome oxidase in both an oxygen-competitive (at heme a3) and oxygen-independent (at Cu) manner. Before inhibition of oxygen consumption, changes can be observed in enzyme and substrate (cytochrome c) redox state. Physiological consequences can be mediated either by direct “metabolic” effects on oxygen consumption or via indirect “signaling” effects via mitochondrial redox state changes and free radical production. The detailed kinetics suggest, but do not prove, that cytochrome oxidase can be a target for NO even under circumstances when guanylate cyclase, its primary high affinity target, is not fully activated. In vivo organ and whole body measures of NO synthase inhibition suggest a possible role for NO inhibition of cytochrome oxidase. However, a detailed mapping of NO and oxygen levels, combined with direct measures of cytochrome oxidase/NO binding, in physiology is still awaited.

mitochondria; cytochrome oxidase

NITRIC OXIDE (NO) is known as an intercellular messenger, synthesized in mammalian systems from arginine, NADPH, and oxygen by the NO synthase (NOS) class of enzymes (1). It has a wide range of physiological functions, most notably the control of blood pressure and blood flow (46, 63) mediated by the production of cGMP via its activation of the heme enzyme, soluble guanylate cyclase (45).

Just over a decade ago, several groups demonstrated that the primary target for NO interactions with mammalian mitochondria is at the level of the oxygen-consuming enzyme, cytochrome c oxidase (15, 20, 73). These, and numerous subsequent studies, have demonstrated that in vitro cytochrome c oxidase is reversibly inhibited by nanomolar levels of NO. The possible cellular consequences of this effect are described in our previous paper (38). This review will instead focus on the importance of NO interactions with cytochrome oxidase at levels of organization above that of the individual cell.

Two major questions arise when contemplating the possible consequences of this interaction at the tissue/organ level. First, does it occur in vivo? Second, if so, what are the physiological consequences? Whereas the detailed molecular mechanism of inhibition might appear to be of more interest to biochemists than physiologists, understanding the chemistry involved informs both physiological and biochemical questions. Several reviews have discussed aspects of this topic (11–13, 17, 23, 24, 26, 37, 59). Here, we attempt an integrated synthesis, in particular by using kinetic modeling to illustrate how the biochemistry can inform the physiology.

THE CHEMISTRY OF INHIBITION

It was shown in 1994 that NO inhibition of cytochrome c oxidase was enhanced at low PO2 (15); the authors suggested that the inhibition was “competitive” in the strict biochemical sense (that is the inhibitor competed with substrate at the substrate binding site). Cytochrome c oxidase has a heme/copper binuclear active site (87). The enzyme only binds oxygen when both these metals are reduced (55, 60), with the iron being ferrous (Fe2+) and the copper cuprous (Cu+) NO binds rapidly (6) and reversibly (71) to the ferrous heme (termed heme a3 in the active site and therefore the suggestion of competitive inhibition seemed biochemically plausible.

The competitive model has endured the rigorous approach of several experimental settings reported in the last decade. Although the inhibition is more effective at lower PO2, this alone does not necessarily equate to pure competitive inhibition. More recent data suggest that NO can be metabolized by oxidized copper (Cu2+) in the active site, forming nitrite (27, 35, 71, 85). Because this form of the enzyme cannot bind oxygen, this results in a mixed type of inhibition. Including this interaction is necessary to model accurately the steady-state kinetics of inhibition (56). Noncompetitive inhibition predominates at low enzyme turnover (when the enzyme is more oxidized) and competitive inhibition at high turnover (when the enzyme is more reduced). It is important to note that, even at low turnover; the inhibition is more effective at low PO2; the effect is just not as pronounced as in the pure competitive model (3). This “dual pathway” model of interactions of NO with cytochrome oxidase is illustrated in Fig. 1.

The discussion above refers to the direct action of oxygen out-competing NO binding. However, it has also been suggested that oxygen can modulate NO inhibition by effects distant from the enzyme-active site. For example, in most biological situations, the metabolic pathways that remove NO...
are oxygen sensitive; this can either be directly via chemical reaction with O$_2$ (53) or indirectly via proteins the NO breakdown activity of which is O$_2$ sensitive, e.g., oxyhemoglobin (54). In vitro, a bolus of NO added to a biological system at low PO$_2$ will result in longer-lasting inhibition than at higher PO$_2$, simply because the NO will decay slower; in vivo, a given steady-state level of NO production, all else being equal, will result in greater inhibition at lower PO$_2$ as it will support a higher steady-state $p$NO. This is especially true in mitochondria where a major pathway for NO breakdown is likely to be the extensive membrane system that concentrates lipid soluble neutral gases such as NO and O$_2$. Thus the NO available to bind to the reduced intermediate of the enzyme is modulated by the O$_2$ concentration surrounding the binuclear O$_2$ binding site buried in the hydrophobic phase of the membrane. (77). Figure 2A illustrates the effect of oxygen and mitochondrial membranes in catalyzing NO removal.

Thus, in principle, oxygen can modulate NO inhibition by acting outside the enzyme active site. However, the effects of oxygen persist when the actual NO steady-state concentration is plotted against flux at different PO$_2$ (Fig. 2B); less NO is required for inhibition at lower oxygen tensions (14, 15, 56). Plots of the type illustrated in Fig. 2B cannot be explained by indirect effects of oxygen catalyzing NO removal from biological systems. This situation can be illustrated with a simple dynamic model of NO interactions with oxygen and cytochrome oxidase (Fig. 3A), NO removal (nonenzymatic) is oxygen concentration dependent (Fig. 3B). Therefore, whether the enzyme is inhibited noncompetitively (Fig. 3C) or competitively (Fig. 3D), the PO$_2$ affects the inhibition at a given time after a NO bolus. However, when the steady-state NO concentration is plotted against the oxygen consumption rate, there is no effect of oxygen concentration when pure noncompetitive inhibition is present (compare Fig. 3, E and F). Some component of direct competition at the enzyme active site is thus required to explain the kinetic data.

In a physiological system the complexities of NO production, as well as decay, need to be considered. The NO production rate is also likely to be oxygen concentration dependent. The enzyme that generates NO, NOS, has both NADPH and oxygen as substrates (1). The PO$_2$ will therefore have direct and indirect effects on NOS activity: direct because NOS has a higher $K_m$ (67) for O$_2$ (5–20 μM) than cytochrome oxidase (<1 μM) and indirect because the steady state level of NADPH is likely to be affected by the PO$_2$. This complicates the situation in vivo as, depending on the details of the in vivo sensitivity of NOS to changes in PO$_2$, it will tend to

Fig. 1. Dual pathway model for the interaction of nitric oxide (NO) with mitochondrial cytochrome oxidase. The oxidized form (ferric/cupric) of cytochrome oxidase cannot react with oxygen. Respiratory substrates (such as pyruvate) inject electrons into the mitochondrial respiratory chain, which ultimately arrive at cytochrome oxidase, the terminal electron acceptor. This reduced enzyme (ferrous/cuprous) can then reduce oxygen to water. NO can bind to the reduced enzyme, generating a nitrite-inhibited oxidized inhibited species; this reaction predominates at low electron flux and is not competitive with oxygen. NO can also react with the oxidized form of the enzyme, generating a nitrite-inhibited oxidized inhibited species; this reaction predominates at high electron flux and is competitive with oxygen. However, NO can also react with the oxidized form of the enzyme, generating a nitrite-inhibited oxidized inhibited species; this reaction predominates at high electron flux and is competitive with oxygen. However, NO can also react with the oxidized form of the enzyme, generating a nitrite-inhibited oxidized inhibited species; this reaction predominates at high electron flux and is competitive with oxygen. However, NO can also react with the oxidized form of the enzyme, generating a nitrite-inhibited oxidized inhibited species; this reaction predominates at high electron flux and is competitive with oxygen. However, NO can also react with the oxidized form of the enzyme, generating a nitrite-inhibited oxidized inhibited species; this reaction predominates at high electron flux and is competitive with oxygen.

Fig. 2. Dependence of NO removal and cytochrome oxidase inhibition on O$_2$ concentration. A: O$_2$ concentration dependence of NO removal in buffer and in the presence of mitochondria. Figure taken from Ref. 77 (Fig. 2A in original publication). B: O$_2$ sensitivity of NO inhibition of oxygen consumption rate. Figure taken from Ref. 77, with permission (shown as Fig. 4A in original publication). Mitochondria were in “state 3” (actively phosphorylating). ○, 80% O$_2$; ■, 30% O$_2$. Copyright 2001 National Academy of Sciences USA.
make the NO/cytochrome c oxidase interaction less effective at low PO2.

THE CHEMISTRY OF NONINHIBITION

Before leaving enzymology to address the in vivo situation, we need to look more closely at how enzymes function outside the in vitro assay. For a range of practical reasons biochemists generally measure enzyme activities as initial rates at fixed substrate concentrations. These original data are usually used to calculate the Michaelis constants, Vmax and Km. However, in vivo, substrates are not fixed entities. In particular, they can vary with the enzyme activity itself. Quantitatively, this can be described in metabolic control theory as the sensitivity of the substrate concentration to enzyme activity (16, 47). For example, adding an inhibitor of oxygen consumption like NO will result in a transient decrease in the rate of oxidation of the substrate, cytochrome c. However, if, as expected, the rate of electron entry (reduction) into cytochrome c from the rest of the mitochondrial electron transport chain were unchanged by NO, an increase in the steady-state level of reduced cytochrome c will ensue. Therefore the first effect of NO inhibition of oxygen consumption by cytochrome c oxidase will be to increase the concentration of the other substrate, reduced cytochrome c. As the flux is proportional to the concentration of reduced cytochrome c, this has the effect of buffering the inhibition of oxygen consumption. The consequence is that the flux through the enzyme will change less than the intermediate redox states (Fig. 4, A and B). The addition of the noncompetitive interaction with the copper site seems to exacerbate this situation with rather large changes in enzyme intermediate concentrations possible for minimal changes in O2 consumption (56). This effect, whereby it is easier to change intermediate and metabolite concentrations than pathway flux, is becoming increasingly recognized in the quantitative analysis of complex biochemical pathways (29).

The consequences for O2 consumption by cytochrome c oxidase have long been recognized by bioenergetics. As first described by Mahaffey and Wilson, in hypoxia cells respond to a drop in O2 by changing the redox state of cytochrome c at a significantly higher PO2 than that required to affect oxygen consumption (91, 93). Similar effects can be seen in vivo during hypoxia in the brain (80). The biochemical consequences of decreasing the substrate concentration are similar to those of adding a competitive inhibitor. Incorporation of changes in the redox state of cytochrome c, as described above, are therefore also required to explain the in vitro and in vivo kinetics of inhibition of oxygen consumption by the pure, O2 competitive inhibitor, carbon monoxide (19). It has recently been shown that the addition of NO has the same effect as hypoxia on the cytochrome c steady state (62). The important consequence of this when studying the interaction of NO in physiology is that effects can occur via changes in redox state of enzyme and pathway intermediates, as well as via a direct effect on oxygen consumption and energy transduction. We can label these as signaling and metabolic consequences of NO interactions with cytochrome c oxidase, respectively. We discuss the possible physiological importance of these two processes later in the review.

THE REALITY OF INHIBITION?

Since the first descriptions of the interaction of NO with cytochrome c oxidase, questions have been raised as to its physiological or pathological relevance. It is clear that the addition of submicromolar levels of NO to mitochondria reversibly inhibit oxygen consumption in isolated mitochondria (7, 15, 18, 20, 73) and cells (14, 18, 62, 72) with kinetic and spectroscopic characteristics identical to those of the purified enzyme (15, 36, 56, 84). The NO can be added exogenously (7, 15, 20, 73), or it can be produced via inducible (14) or constitutive (21) levels of NOS in the cell culture. The appropriate cellular physiological consequences—collapse of mitochondrial membrane potential (73), inhibition of ATP synthesis (9), and activation of glycolysis (2)–ensue. Indeed, even if this interaction was to prove physiologically unimportant, its ability to explain the artifacts seen when high levels of NO are added, rather indiscriminately, to cells would be a useful addition to the scientific literature.

However, clearly it would be even more interesting if NO was proven to be a physiological regulator (in which case it would be the first external regulator of the mitochondrial respiratory chain). The skepticism of some as to the physiological relevance of inhibition at cytochrome c oxidase may be related to a general reluctance to accept that an enzyme at the end of a metabolic pathway can control flux through that pathway (as it is beyond the “committed step”). In one sense the breaking of the oxygen-oxygen bond by cytochrome oxidase is the “committed step” in the mitochondrial electron transfer chain as it is the only one that is irreversible under physiological conditions. However, even if this were not so, control is still possible at the end of a pathway because changes in the level of intermediates “communicate” through the whole pathway. If control of flux is defined as the fractional change in pathway flux for a fractional change in activity of that enzyme (as in metabolic control analysis), it can be shown that there is no unique step that controls flux in the steady state. Instead many enzymes contribute to a distribution of control (33) with 1 being total control and 0 being no control. Under several conditions, cytochrome c oxidase has significant nonzero control over mitochondrial oxygen consumption (81). Under these conditions rather small changes in the enzyme activity can affect the oxygen consumption rate. Of course high concentrations of any inhibitor will eventually inhibit pathway flux, whatever the initial control in that enzyme or its position in the pathway.

What is the evidence for the effects in vivo? Unfortunately it is almost impossible to make quantitative in vivo predictions from the in vitro calculated inhibition constants as the in vivo mitochondrial pNO and PO2 at cytochrome oxidase are very poorly characterized. Therefore direct in vivo measurements of the NO/oxidase interaction are required. If in vivo means in cell culture, then, as noted above, the case is very strong. However, these cellular measurements may not be readily applicable to physiology; here NO scavenging systems may be present that are not generally present in cell culture (e.g., oxyhemoglobin) and, in many cell types, activation of glycolysis can mask an inhibition of mitochondrial energy production (the Pasteur effect).

If NO was inhibiting mitochondrial oxygen consumption in vivo, then, all else being equal, the addition of NOS
inhibitors might be expected to increase oxygen consumption. A wide range of studies from Thomas Hintze’s group (reviewed in Ref. 86) confirm this for tissue slices from cardiac muscle (95), skeletal muscle (74), and kidney (52). Studies in the conscious dog using NOS inhibitors have shown increases in oxygen extraction in the heart (5), skeletal muscle (48), and whole body (75). These effects persist even when the effects of NO on blood flow are taken into account. However, NOS inhibitors do not always seem to increase myocardial oxygen consumption (42, 49, 68, 76); the reasons for this discrepancy are not clear, although in some cases they are likely to relate to differences in the intracellular location, local concentration, permeability and \( K_i \) of different inhibitors. Interestingly, no effect has been seen of NOS inhibitors on either oxygen consumption (44, 69) or the redox state of cytochrome oxidase (30) in the anesthetized brain, even in the absence of potentially NO scavenging red blood cells (90).

The main problem with in vivo studies is that it is not easy to determine whether the effects on oxygen consumption are mediated by cytochrome oxidase rather than indirectly by other NO signaling pathways (primarily of course activation of soluble guanylate cyclase). Studies with the cGMP analogue, 8-Br-cGMP, are not always able to completely reverse the effects of NOS inhibitors (32), consistent with a mitochondrial explanation for at least some of the in vivo effects.

It is clear that the evidence is highly suggestive of an in vivo interaction between NO and cytochrome c oxidase, but some key in vivo experiments remain to be done. Magnetic resonance spectroscopy measures of mitochondrial energetics (28) would complement the whole body oxygen consumption data.
described above, putting the primary site of action squarely at the mitochondrion. Direct measures of the cytochrome c or cytochrome c oxidase redox states are possible in vivo using optical techniques, although in whole animals the ability to deconvolute cytochrome c oxidase signals from the hemoglobin chromophore present at higher concentrations is still controversial (25, 57). The only place where these have been tried is in the brain (30), where we have already noted that there is no strong evidence of NO effects on oxygen consumption.

**NO Inhibition Compared with Guanylate Cyclase Activation**

Are the NO concentrations that inhibit cytochrome c oxidase likely to have physiological relevance compared with those required for guanylate cyclase inhibition? Garthwaite has made this comparison (4), and a modified version of these data is presented in Fig. 5. Given the fact that guanylate cyclase has an affinity for NO of ~4 nM, inhibition constants of 120 nM for cytochrome c oxidase mean that, whenever cytochrome c oxidase is inhibited by NO, its main signaling pathway will already be 100% active. The data analyzed by Garthwaite were from cerebellar cells, and the brain is the organ where there is least evidence in vivo for NO/cytochrome c oxidase interactions (see above). Nevertheless the implications of this result is relevant when discussing physiological roles of NO inhibition of the oxidase (the problems is less acute for pathology, e.g., immune responses, where levels of NO are much higher).

There are several valid responses to the challenge posed by data of this kind. First, there is the argument based on compartmentalization. If cytochrome c oxidase and guanylate cyclase sense different levels of NO then the graph is not relevant. Microheterogeneity, caused, for example, by NOS binding to cytochrome oxidase is discussed in our recent review (38). However, even were the proteins to be exposed to the same NO concentrations, the details of the analysis could be modified by cellular conditions. A PO2 of 30 μM is an

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**Fig. 3—Continued**

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**Fig. 4. Buffering of inhibition of cytochrome oxidase activity by changes in cytochrome c redox state.** A: model scheme is broadly based on that of Antunes model (3), assuming the rapid reversible binding of a competitive inhibitor (I) to cytochrome oxidase and a relatively slow, limiting flux for cytochrome c reduction by the respiratory chain. Rate constants, though physiologically feasible, were primarily chosen to illustrate the points made in the text, rather than to model a specific situation in the cell. $k_1 = 1 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$, $k_2 = 4 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$, $k_3 = 5 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$, $k_4 = 1 \text{ s}^{-1}$, and $k_5 = 0.1 \text{ s}^{-1}$. For kinetic simulations the initial enzyme concentration (E) was set to 5 nM, the initial cytochrome c to 2 μM and the oxygen concentration to 230 μM. B: kinetic effect of adding an inhibitor on the cytochrome c redox state and the flux. The enzyme was allowed to enter a steady state and then 10 μM inhibitor added (at $t = 0$).
average for the whole brain; many parts of the brain will, by
definition, have a lower PO2. Other tissues will have still lower
PO2, e.g., the myocardium (22, 34). Furthermore, the gradient
of PO2 between the tissue and cytochrome c oxidase is still
controversial (82, 94). Some gradient, however, shallow, must
exist, and the steeper it is, the lower the PO2 at the enzyme and
the higher affinity the inhibition will be.

Figure 5 compares what is essentially a binding curve
(guanylate cyclase activation) with an inhibition curve
(oxygen inhibition). The latter is modified by other factors in the cell
(principally how much control the O2 reactions at the enzyme
active site has over cellular oxygen consumption under the
conditions assayed). At lower O2 consumption rates NO is a
very poor inhibitor or signaler (requiring micromolar concen-
trations) (3, 56) and “right shift” occurs. Conversely at higher
fluxes the NO inhibition becomes more significant resulting in
a “left shift”. Figure 5 illustrates how relatively small changes
in cellular PO2 or enzyme Km can result in significant overlap
between the levels of NO required for guanylate cyclase
activation and cytochrome oxidase inhibition.

As the apparent Km for oxygen is a kinetic parameter
dominated by the rate of electron transfer to the enzyme active
site, not the binding affinity of oxygen per se (88), it is possible
to envisage situations where the Km for oxygen can change
dramatically with little change in NO binding equilibria.
Therefore factors that increase the Km for oxygen will,
by definition, increase the potency of a broadly competitive
inhibitor such as NO (as NO can then more effectively
compete with oxygen). This effect has been best character-
ized by the effect of increasing the rate of electron transfer to
the enzyme via increases in the cytochrome c redox state. This
has the effect of increasing the oxygen Km and decreasing the
IC50 (3, 56) These points are illustrated in Fig. 6. This might be
the reason why in rat aortic rings, where the oxygen Km was
measured to be as high as 30 μM, NO was seen to perturb
oxygen consumption rates when guanylate cyclase was still
only partially activated (61).

Finally, as discussed earlier, the interaction of cytochrome c
oxidase with NO does not require inhibition of O2 consumption;
mitochondrial redox state changes can be significantly left shifted
from the curve measuring inhibition of O2 consumption (56, 62,
91). Thus, in vivo a plot of redox state of cytochrome oxidase (or
cytochrome c) vs. guanylate cyclase activation will be “left
shifted” compared with similar plots of O2 consumption.

The combination of these factors demonstrates that NO can,
at least in theory, signal via cytochrome oxidase without
maximal activation of guanylate cyclase. However, the com-
plexity of the interactions requires that measurements under
identical cellular physiological conditions are required to con-
firm these effects.

Physiological Relevance of NO/Cytochrome
Oxidase Interactions

Mitochondrial cytochrome c oxidase is a key enzyme in
mammalian systems, the absence of which in incompatible
with life. Inhibitors of enzyme activity (e.g., cyanide) are

![Fig. 5. Dependence of [NO] on soluble guanylate cyclase and cytochrome oxidase activities. The plot illustrates some of the kinetic factors that can modify the relationship between these two NO-related activities. It assumes an EC50 of 4 nM NO for the activation of soluble guanylate cyclase (sGC) and a K_i (app) of 120 nM NO for the inhibition of cytochrome oxidase at 30 μM oxygen (values taken from Ref. 4). Plots at 30 μM oxygen from this paper are contrasted with simulations at 10 μM oxygen, and at 10 μM oxygen but also using a 3× higher Km of oxygen for cytochrome oxidase. Pure competitive inhibition is assumed. Note that the shape of these kinetic “Hill type” plots do not directly inform on the number of NO molecules binding to cytochrome oxidase: a statement that was incorrectly attributed to the authors of Ref. 4 by us in Ref. 56. For comparison purposes values of 1 for the Hill coefficient were used in all cases.

![Fig. 6. Dependence of IC50 for NO on the turnover of cytochrome oxidase. In a simple model of cytochrome oxidase kinetics, the oxygen Km is directly related to the enzyme turnover when the latter is varied by increasing the concentration of the other substrate, reduced cytochrome c. This plot illustrates this effect and the consequent effect on the IC50 for NO inhibition. The Km is obtained by dividing the enzyme turnover (TN in electrons per second) by the apparent oxygen “on rate” (itself a complex mixture of binding and electron transfer rates): Km(O2) = TN(s^-1)O2 on(M^-1 s^-1). Assuming pure competitive inhibition the IC50 for NO can then be calculated by the standard enzyme kinetics equation IC50 = Km(1 + [O2]/Km(O2)). See Antunes et al. (3) for an expanded discussion of this. Parameters used in this plot: [O2] = 30 μM; O2 on = 1.4 × 10^8 M^-1 s^-1.](http://ajpcell.physiology.org/10.2203033)
highly toxic. So, why would nature have evolved a signaling mechanism involving inhibiting the main energy-transducing pathway of the cell? One answer is to suggest that the enzyme has evolved not to be inhibited. Indeed, we have argued previously (24) that given the ubiquity of NO signaling in biology and its structural similarity to oxygen, all metalloproteins that react with oxygen may have evolved mechanisms to prevent being inhibited by NO. The off rates for NO from ferrous cytochrome oxidase are, for example, significantly higher than those for many heme proteins (e.g., hemoglobin, myoglobin), explaining why inhibition when it is observed is readily reversible. Nevertheless there have been numerous hypotheses describing a physiological role to the interaction of NO with cytochrome c oxidase (12). These can be divided into two broad categories: those where the inhibition of oxygen metabolism itself is the key event and those where the NO/oxidase interaction interfaces with other signaling pathways. These ideas are summarized in Table 1.

WHY INHIBIT O2 CONSUMPTION IN PHYSIOLOGY?
THE METABOLIC ARGUMENT

A direct effect of NO on renal oxygen consumption has been suggested (52) to play a role in regulating the balance between sodium reabsorption (ATP utilizing) and oxygen consumption (ATP generating). The amount of oxygen required for sodium reabsorption significantly increased (by 160%) after the addition of a NOS inhibitor. This implies that NO makes mitochondrial ATP generation more efficient, though it is unclear how this could act via a direct effect on cytochrome oxidase (P/O ratios normally decline in the presence of respiratory inhibitors). The physiological role of NO in inhibiting cardiac oxygen consumption is not clear. However, this effect is attenuated in diabetes or cardiomyopathy, suggesting it may have a protective effect (86). This may be related to the idea that the depression of mitochondrial metabolism in septic shock, where NO is overproduced, may have a protective role (8, 79). It is intriguing that the turtle brain, which is highly resistant to hypoxia, has an almost linear dependence of cytochrome oxidase redox state on a reduction in oxygen delivery, as opposed to the “threshold” effect seen in mammals (78); this implies that cytochrome oxidase may always be oxygen-limited in the turtle, as might be expected were NO to be present at levels to inhibit the enzyme.

The physiological mechanism suggested to underlie these effects is not well characterized. However, more detailed dynamic molecular mechanisms have been proposed for NO effects on oxygen metabolism in the vasculature and in the brain. Lancaster has developed a model where NO produced at the endothelium diffuses away from the vessel (83). Including the ability of NO to inhibit oxygen consumption has the effect of making the oxygen gradient to cells distant from the blood vessels much shallower. These same cells have a lower NO content (as the steady-state NO concentration decreases from its point of formation). This higher O2 and NO content significantly increases O2 consumption rates at cells distant from the vessel. It is important to note that as the primary NO-dependent event modeled is an inhibition of oxygen consumption, the overall effect (whatever the change in the O2 and NO gradients) must also be to decrease oxygen consumption. Therefore, this can only be of benefit physiologically if the cells distant from the vessel “gain” more than those near the vessel “lose”. One possibility is that there are critical ATP levels below which cells cannot function; in this case dropping ATP synthesis by a small amount in many cells is an acceptable sacrifice if it allows a few cells to remain above this critical threshold.

The modeling in the study described above focuses on the development of gradients from an identical NO and oxygen source to distance cells that do not produce either gas (the “classic” intercellular signaling mode for NO). The possibility of intracellular NO/oxygen gradients between, for example, mitochondrial NOS and cytochrome oxidase cannot be discounted, and this issue has been discussed before in our earlier review (38).

Finally, Gjedde (39, 40) has proposed a key role for NO inhibition of cytochrome c oxidase in explaining flow-metabolism coupling in the brain. In this model, Gjedde assumes that an increase in flow will increase the O2 gradient between the capillary and the mitochondria. Therefore, all else being equal, there should be an increase in oxygen consumption (depending on the sensitivity of mitochondrial O2 consumption to these PO2 changes). In many cases, however, this is not seen. The author argues that to explain this anomaly it is necessary to assume that an increase in flow induces a decrease in the affinity for cytochrome oxidase for oxygen. An increase in cerebral blood flow is generally accompanied by (or in many cases caused by) an increase in NO levels; therefore NO inhibition of cytochrome c oxidase, competitive with oxygen, is an intriguing mechanism to explain these findings. However, the model also requires that cytochrome c oxidase metabolizes NO in an oxygen concentration dependent manner. The experimental evidence for this is limited but it is possible that the metabolism of NO by mitochondrial membranes [which is strongly O2 concentration dependent (53, 77)] might be a suitable alternate mechanism.

Table 1. Suggested biochemical and physiological consequences of nitric oxide interactions with cytochrome c oxidase

<table>
<thead>
<tr>
<th>Interactions</th>
<th>Reference No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acting directly via effects on O2 metabolism</td>
<td></td>
</tr>
<tr>
<td>Regulation of balance between renal sodium reabsorption and O2 consumption.</td>
<td>52</td>
</tr>
<tr>
<td>Protective effect in myocardium via inhibition of cardiac O2 consumption.</td>
<td>86</td>
</tr>
<tr>
<td>Extending O2 gradient from blood vessel to more distant cells.</td>
<td>83</td>
</tr>
<tr>
<td>Controlling flow metabolism coupling in the brain</td>
<td>39, 40</td>
</tr>
<tr>
<td>Moderating HIF hypoxia sensing by raising intercellular PO2.</td>
<td>43</td>
</tr>
<tr>
<td>Activation of glycolysis</td>
<td>2</td>
</tr>
<tr>
<td>Acting indirectly via other signalling pathways</td>
<td></td>
</tr>
<tr>
<td>Activation of redox-sensitive signal transduction pathways.</td>
<td>32</td>
</tr>
<tr>
<td>O2 sensing in carotid body</td>
<td>12</td>
</tr>
<tr>
<td>Hypoxia sensing in endothelial cells</td>
<td>32</td>
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<tr>
<td>HIF, hypoxia-inducible factor.</td>
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SIGNALING VIA CYTOCHROME C OXIDASE

NO/cytochrome c oxidase interactions have been suggested to be involved in a range of signaling pathways. Even prior to the discovery that NO reversibly inhibited cytochrome c ox-
dase, the enzyme was suggested to be involved in oxygen sensing (58). The primary oxygen sensor of vascular Po2 is the carotid body (41). The fact that carbon monoxide can mimic low O2 in activating this pathway, and that the photoaction spectrum of this activation closely resembled that of CO dissociation from cytochrome c oxidase, suggested that the primary oxygen sensor could, in fact, be the cytochrome c oxidase itself (50, 92). One concern with this mechanism is that the K_m for oxygen for the enzyme might too low to be able to respond to the small changes from physiological Po2 that the carotid body detects. However, as stated previously in this review not only is the Po2 at the mitochondria a matter of current debate, but enzyme redox state changes can occur at higher Po2 than oxygen consumption changes. In any case, it is clear, as originally suggested by Brown (12), that the presence of NO can theoretically allow CCO to detect O2 changes in the physiological sensing range. Multiple transmitters with differing O2 affinities have been proposed to explain the ability of the carotid body to detect O2 in the range from 80 to 20 mmHg (64). Combinations of O2-sensitive heme enzymes (cytochrome oxidase, heme oxygenase, NOS) and O2 sensitive ion channels would combine to make this “chemosome.” An intriguing alternative to multiple sensors would be a single sensor capable of being modified by its environment. Cytochrome oxidase in the presence of varying concentrations of NO (in different cell types?) could clearly sense over this wide dynamic range. The presence of NO would allow any putative oxidase sensing mechanism to be tuneable over a very wide range of Po2.

Tuning of hypoxia sensing by NO inhibition of respiration can also occur intracellularly via interactions with other O2 sensors. NO inhibition of consumption will raise cellular Po2, such that other sensors become less sensitive to a decrease in external Po2. This has been shown for hypoxia inducible factor, where dropping the Po2 in a cell culture medium to 1% results in O2 sensing in the absence of NO, but not in its presence (43). Clearly, even more so than in the “vascular O2 sparing” hypothesis, such a sensing system can have deleterious consequences for the energetics of the cell. In vivo any such mechanism is therefore likely only to be present in cells that can maintain main cellular functions through ATP provided by glycolysis alone (65).

Fig. 7. Mitochondrial reactive oxygen species (ROS) in signal transduction. The incomplete reduction of O2 at the electron transport chain results in the formation of superoxide anion. Superoxide dismutase catalyzes the dismutation of this anion to hydrogen peroxide. Increased ROS may increase the level of oxidatively damaged biomolecules, such as nucleic acids, lipids, and proteins. In addition, ROS may activate downstream redox-sensitive signal transduction events. In this latter category we can include NF-κB, PKC, p30 MAPK, among others. ROS may interfere with iron-dependent pro-hydroxylases, promoting the heterodimerization of hypoxia-inducible factor (HIF) and its subsequent transcriptional activity [more details of the HIF mechanism are given in Fig. 2 of our previous review (38)].
How can the primary event (NO binding to cytochrome oxidase) be communicated to a signaling pathway? The suggested mechanism is via modulating mitochondrial oxygen radical production (superoxide) and hence changing peroxide concentrations in the cell. This would transduce the mitochondrial signal to a wide range of peroxide-sensitive signaling pathways (e.g., p38, JNK, NF-κB, and PKC) (10) (Fig. 7).

What is the mechanism for the NO perturbation of radical production? The literature on mitochondrial oxygen radical production is highly contentious and not particularly consistent. Complications arise because the key superoxide generating reactions are those between flavin and coenzyme Q semiquinone radicals and oxygen, not those at cytochrome oxidase itself. These reactions are linearly dependent on PO2, but the steady-state concentrations of the relevant radicals are highly dependent on the ubisemiquinone steady-state concentration, electron flux, the mitochondrial membrane potential, and antioxidant enzymes, e.g., superoxide dismutase, catalase, glutathione peroxidase, and others (89). Complex III is the most important physiologically (31) but complex I can also contribute to this production in certain tissues (51). Can changes in cytochrome oxidase activity perturb these reactions? The inhibition of cytochrome c oxidase with, for example, cyanide does not increase radical production (66). However, despite this, a fraction of the oxygen radical production in mitochondria does seem to be modulated by NO (70), consistent with this NO mitochondrial signaling hypothesis. Cyanide and NO are expected to have similar effects on the global cytochrome c redox state. However, they have very different active site chemistry and distinct on and off rates for cytochrome oxidase molecules, a difference that is presumably communicated to the mitochondrial radical producing sites by an as yet undetermined mechanism.

In conclusion, the interplay between NO and mitochondria is increasingly seen as a key physiological signaling phenomena (at low rates of NO production) and pathological phenomena (at high rates of NO production). Many, if not all, of these reactions are likely to be via interaction with the oxygen consuming enzyme cytochrome c oxidase. Direct biochemical effects can be mediated via perturbations of oxygen consumption rates and indirect biochemical effects via changes in mitochondrial free radical production. However, the consequences of these interactions are likely to vary between cell types, organs, and species. It is now appropriate, and indeed necessary, to try and tie the cellular biochemistry to whole organ and whole body physiology.

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NITRIC OXIDE AND MITOCHONDRIAL OXYGEN CONSUMPTION


