Nitric Oxide, Cytochrome C Oxidase, and the Cellular Response to Hypoxia

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Abstract—Cytochrome c oxidase (CcO; complex IV of the mitochondrial electron transport chain) is the primary site of cellular oxygen consumption and, as such, is central to oxidative phosphorylation and the generation of adenosine-triphosphate. Nitric oxide (NO), an endogenously-generated gas, modulates the activity of CcO. Depending on the intracellular oxygen concentration and the resultant dominant redox state of CcO, the interaction between CcO and NO can have a range of signaling consequences for cells in the perception of changes in oxygen concentration and the initiation of adaptive responses. At higher oxygen concentrations, when CcO is predominantly in an oxidized state, it consumes NO. At lower oxygen concentrations, when CcO is predominantly reduced, NO is not consumed and accumulates in the microenvironment, with implications for both the respiratory rate of cells and the local vascular tone. Changes in the availability of intracellular oxygen and in the generation of reactive oxygen species that accompany these interactions result in cell signaling and in regulation of oxygen-sensitive pathways that ultimately determine the nature of the cellular response to hypoxia. 

(Key Words: nitric oxide ■ reactive oxygen species ■ cytochrome oxidase ■ hypoxia ■ mitochondria)

Interaction Between Nitric Oxide and Cytochrome C Oxidase

Mitochondria produce ATP constantly and are therefore permanently active. The proton gradient that drives the ATP synthase (which generates the majority of ATP normally used by cells) is generated by the transfer of electrons along the respiratory (electron transport) chain. The components of the chain are therefore constantly switching from their oxidized properties of oxygen but could use them in the generation of energy through catalyzing the efficient oxidative metabolism of sugars, such as glucose, and fatty acids.2,3 The key to this development was the incorporation of oxygen-consuming bacteria into the host cytoplasm, leading to the evolution of what have become mitochondria. Such was the increase in efficiency afforded by the use of oxygen as a substrate for the production of adenosine-triphosphate (ATP) that it enabled the rapid development of multicellular forms of life that were totally dependent on the utilization of this gas for some of their key metabolic processes.4 It is not surprising, therefore, that during the course of evolution, molecular mechanisms have developed to respond to low oxygen concentrations [O2] with the induction of a transcriptional response directed toward hypoxic adaptation. It has recently become clear that mitochondria play a critical role in determining the activation of this response.

Oxygen arose in the earth’s atmosphere some 2.3 billion years ago as a by-product generated by photosynthesizing cyanobacteria in the oceans of the planet.1 Hypotheses suggest that the accumulating presence of this colorless, odorless, but highly reactive molecule initially represented a major threat to life on earth. However, an endosymbiotic relationship that developed between 2 primitive cell types during this time led to the evolution of a unicellular organism which was not only resistant to the oxidizing properties of oxygen but could use them in the generation of energy through catalyzing the efficient oxidative metabolism of sugars, such as glucose, and fatty acids.2,3 The key to this development was the incorporation of oxygen-consuming bacteria into the host cytoplasm, leading to the evolution of what have become mitochondria. Such was the increase in efficiency afforded by the use of oxygen as a substrate for the production of adenosine-triphosphate (ATP) that it enabled the rapid development of multicellular forms of life that were totally dependent on the utilization of this gas for some of their key metabolic processes.4 It is not surprising, therefore, that during the course of evolution, molecular mechanisms have developed to respond to low oxygen concentrations [O2] with the induction of a transcriptional response directed toward hypoxic adaptation. It has recently become clear that mitochondria play a critical role in determining the activation of this response.
The discovery that nitric oxide (NO, a gas that closely resembles oxygen structurally) is an endogenous mediator,\textsuperscript{6} together with the later demonstration in vitro experiments of the reaction between NO and oxygen at the CcO and the fact that this enzyme has a greater affinity for NO than for oxygen\textsuperscript{7–9} suggested that this interaction might be physiologically relevant. In spite of some controversy\textsuperscript{10} there is increasing evidence to suggest that NO and oxygen do interact physiologically at the level of the CcO.\textsuperscript{11,12} The nature and consequences of this interaction are dependent on the redox state and turnover of CcO, local oxygen concentrations, and the activity of the NO synthase.\textsuperscript{10,13,14}

Unlike oxygen, NO binds to both the reduced and the oxidized oxygen-binding site of the enzyme\textsuperscript{15} (Figure). When the enzyme is reduced NO binds to the heme $a_1$ in its ferrous state in a manner that is competitive with oxygen; when the CcO is in its oxidized state, however, NO binds not to the oxidized (ferric) heme $a_1$ but instead to the oxidized form of the CuB center in a manner that is not competitive with oxygen. Both of these reactions in cell systems can be considered reversible because the reaction of NO with CcO in its reduced form can be reversed by oxygen, whereas that with the oxidized form leads to the conversion of NO to nitrite (NO$\textsuperscript{2−}$), which then dissociates from the enzyme.\textsuperscript{15} Therefore, a key difference between these 2 distinct interactions of NO and CcO is that in the former (when CcO is reduced) NO is not consumed by the reaction whereas in the latter (when CcO is oxidized) NO is oxidized to nitrite and therefore NO consumption occurs.

A number of routes have previously been proposed for the inactivation of NO in vivo, including chemical interactions with endogenous molecules such as superoxide, hemoglobin, and myoglobin.\textsuperscript{16–18} Further proposed mechanisms for enzymatic NO inactivation have suggested roles for cyclooxygenase, peroxidase, catalase, and a flavo-hemoglobin–like NO dioxygenase.\textsuperscript{19–23} CcO may, however, be a more significant determinant of the local bioavailability of NO, especially as the cell respires toward low [O$_2$]. Indeed, it has been demonstrated that CcO, when it is in turnover and in its oxidized state, inactivates physiological amounts of NO, thus regulating its intra- and extracellular concentrations.\textsuperscript{24,25} This inactivation can be prevented by blocking the enzyme with inhibitors, including NO itself. Furthermore, when cells generating low concentrations of NO were allowed to respire toward low [O$_2$] the redox state of the enzyme changed from oxidized to reduced, leading to a decrease in NO inactivation. Thus, at high [O$_2$], CcO acts as a metabolic sink for NO; however, as the [O$_2$] decreases and the enzyme becomes reduced this action of CcO diminishes.\textsuperscript{24} The rate of generation of NO is critical, because the capacity of the CcO for NO metabolism has a limit (at present not established) beyond which NO will “overflow” and inhibit the enzyme. A corollary of this is that it is likely that at certain rates of synthesis NO is completely metabolized and that the NO detected by the soluble guanylate cyclase is the product of the balance between its synthesis and destruction.

Partial inhibition of CcO by NO leads, in the first instance, to a compensatory mechanism (dependent on the well-known spare capacity of the enzyme\textsuperscript{26}) whereby previously inactive enzyme is recruited to maintain the electron transfer and rate of oxygen consumption. Only when the spare capacity is completely used do electrons start to accumulate in the CcO. There is then a progressive reduction of the enzyme attributable to the backlog of accumulating electrons, leading ultimately to a reduction of the whole respiratory chain. Thus the inhibitory action of NO on the CcO does not initially result in inhibition of oxygen consumption. Only as the NO concentration increases, or that of oxygen decreases, does concentration-dependent inhibition of respiration actually occur.

Because CcO is the crucial enzyme in the process of oxygen consumption it has long been suspected to be involved in oxygen sensing.\textsuperscript{27} The problem with this suggestion has always been that the Km for the isolated enzyme is too low to allow it to “sense” the small variations in oxygen concentration that occur in the body. Interestingly, the measured Km of the CcO for oxygen in various cell types is 20 to 30 times higher than that of the isolated enzyme.\textsuperscript{28} This has led to the idea that it is the interaction between NO and the CcO, which actually elevates the Km of the enzyme for oxygen, that allows the oxygen-sensing function of the enzyme. Such sensing might be tissue-specific and depend on the relative concentrations of NO and oxygen, as well as the activity of the CcO.

In summary, in biological systems there is a range of interactions between NO and oxygen at the CcO. These have a variety of consequences which, as they become understood, throw light onto some aspects of cell signaling, both in homeostasis and during stress conditions. We will try to explain some of these in the following sections.

**Consequences for Tissue Oxygen/NO Homeostasis**

**NO Accumulation**

As cells respire toward low [O$_2$] the proportion of CcO in the reduced state will tend to increase. As already described, this will lead to a decrease in the catabolism of NO, which occurs when the enzyme is in its oxidized state. As a consequence...
NO now becomes an inhibitor of the enzyme, in competition with oxygen. Recent evidence suggests that at low $[O_2]$ NO will activate the soluble guanylate cyclase to produce vasodilation and therefore increase the local supply of oxygen. This effect has been suggested to be a significant contributing factor in hypoxic vasodilation.24 Nitric oxide therefore behaves as a rheostat for respiration and eventually acts to ration the consumption of the limited oxygen available. As such, elevated NO resulting from its decreased inactivation by CcO at low $[O_2]$ is a protective mechanism against tissue hypoxia and therefore sets the scene for the development of the response of tissues to this deleterious condition.

### Oxygen Redistribution and HIF Stabilization

Decreased $[O_2]$ has profound effects on gene transcription through activation of the ubiquitously-expressed dimeric hypoxia-inducible factor (HIF), an oxygen-dependent transcription factor consisting of an oxygen-labile α subunit and a constitutively expressed β subunit. HIF activates the expression of a cohort of genes involved in the adaptation of tissues to hypoxia; these include angiogenic, vasoactive, metabolic, and hematopoietic factors. The oxygen sensitivity of the HIF pathway is conferred by a family of oxygen-sensing hydroxylases that includes 3 prolyl hydroxylases (PHD1–3) and one asparagine hydroxylase (factor inhibiting HIF; FIH).29 The PHDs target the HIF-α subunit for degradation through the oxygen-dependent hydroxylation of two residues (Pro402 and Pro564 on the HIF-1α isoform), which leads to its degradation through the ubiquitin/proteasome pathway. Furthermore, in the presence of available intracellular oxygen, FIH confers oxygen-dependent transcriptional repression through asparagine hydroxylation (Asp803 on HIF-1α) and one asparagine hydroxylase (factor inhibiting HIF; FIH).29 The PHDs target the HIF-α subunit for degradation through the oxygen-dependent hydroxylation of two residues (Pro402 and Pro564 on the HIF-1α isoform), which leads to its degradation through the ubiquitin/proteasome pathway. Furthermore, in the presence of available intracellular oxygen, FIH confers oxygen-dependent transcriptional repression through asparagine hydroxylation (Asp803 on HIF-1α), an event that prevents the binding of the CREB binding protein (CBP) and p300 coactivators and thus represses initiation of the transcriptional complex formation. Thus, when sufficient oxygen is available, the oxygen-dependent hydroxylases suppress HIF-1α stability and activity. In hypoxia, however, the hydroxylases are nonfunctional, leading to HIF-1α stabilization and transactivation.30

Inhibition of mitochondrial respiration by NO at low $[O_2]$ has been shown to result in redistribution of oxygen toward nonrespiratory oxygen-dependent targets, including PHDs, thus modulating the response of tissues to hypoxia. Nitric oxide therefore acts as an endogenous regulator of the intracellular availability of oxygen in mammalian cells.31,32 Such destabilization of HIF-1α has been shown not to be dependent on the generation of reactive oxygen species (ROS) by mitochondria because it is not affected by antioxidants31,33,34 or by addition of $H_2O_2$.35 Furthermore, inhibitors that act at different points in the respiratory chain, and therefore have different effects on the generation of mitochondrial ROS, all prevent accumulation of HIF-1α during hypoxia.31,36

Recent evidence indicates that inhibition of the CcO might also play a role in the adjustment of tissue oxygen consumption at low $[O_2]$. Measurement of oxygen consumption in blood vessels in a hypoxic chamber, and oxygen distribution in the microcirculation using a fluorescent oxygen-probe, has demonstrated that endogenously-released endothelial NO, either basal or stimulated, can modulate oxygen consumption both throughout the thickness of conductance vessels and in the microcirculation.37

### Generation of ROS

Because of their strategic role in oxygen use, the mitochondria and their respiratory chain enzymes are likely to be central to any mechanism of generation of ROS and their signaling consequences. In spite of this, the demonstration of ROS generation from mitochondria remains controversial, especially in hypoxia.38 One of the main difficulties relates to the absence of generally agreed methodologies to measure ROS, especially in vivo. The other difficulty is based on the fact that many biochemical processes are potentially generators of these species.39 This has led, in the last few years, to an increasing body of evidence suggesting that mechanisms other than mitochondria might be involved in the generation of ROS for cell signaling, including NADPH oxidases, xanthine oxidase, and uncoupled eNOS.39

In relation to mitochondria, early evidence, mainly from studies using the isolated organelle, suggested that a small percentage of the oxygen used by the electron transport chain is not completely reduced to water but is instead converted to superoxide anion [$O_2^-$. This occurs as a result of the escape of electrons at complexes I and III of the chain.40–44 More recent experiments support the view that the release of ROS from mitochondria may indeed occur in hypoxia45 and that this is dependent on complex III.46

Studies using visible light spectroscopy have shown that generation of ROS occurs at low $[O_2]$ and is dependent on the redox state of the electron transport chain which, as described above, is regulated by NO acting on the CcO. Thus ROS generation might also be modulated by the interactions between NO and oxygen and might occur not strictly speaking in hypoxia but whenever the [NO] increases in relation to the $[O_2]$ to a point at which reduction of the chain occurs.47 Ultimately, in hypoxia, although the electron transport chain is reduced there may not be enough oxygen for the generation of ROS.

### ROS Signaling

Signaling by ROS has been suggested to activate a number of transcription factors, including nuclear factor kappa B (NF-κB), activator protein 1 (AP-1), specificity protein 1 (Sp1), peroxisome proliferator-activated receptors (PPARs), and other members of the nuclear receptor superfamily.48

The NF-κB pathway has been shown to be activated by hypoxia and to contribute to hypoxic inflammation.49 Furthermore, NF-κB signaling has been demonstrated to be central to the HIF pathway and is thus critical in the overall cellular response to low $O_2$.50,51 The link between ROS signaling and NF-κB activity is controversial.52 Mitochondrial ROS production in hypoxia has been suggested as a possible signaling link between hypoxia and NF-κB.47,53 However, recent studies provide evidence that the oxygen sensitivity of the NF-κB pathway, like HIF, is conferred by oxygen-dependent hydroxylases,54 and thus molecular oxygen availability may be the key signal for hypoxia-induced NF-κB activation.
The enzyme AMPK has, until recently, been considered mainly as a sensor of the bioenergetic status of cells, which responds to increases in AMP by adjusting their metabolism to conserve the concentration of ATP. Recent studies have demonstrated, however, that AMPK can be activated through other mechanisms, including Ca\(^{2+}\)/calmodulin kinase kinase II\(^{56,57}\) and signaling by ROS.\(^{58,59}\) Furthermore, in human vascular endothelial cells mitochondria-generated ROS activate the enzyme in the absence of changes in AMP concentration, suggesting that its activation might be involved in functions beyond bioenergetic adaptation.\(^{59}\) Indeed, it has been suggested that mitochondrially-derived ROS, by activating a specific variant of the AMPK that is abundant in the vascular endothelium, might not only trigger an antioxidant defense response but also act as a mechanism to maintain a physiological basal antioxidant tone.\(^{60,61}\)

**ROS and Stabilization of HIF**

Although the stabilization of HIF-1α is likely to be determined mainly by the concentration of oxygen regulating the activity of the PHDs,\(^{70}\) there is a significant body of evidence indicating that ROS are also able to stabilize this transcription factor. It was originally suggested that ROS generated in hypoxia were responsible for this action,\(^{44}\) and in recent years further evidence has been produced to support this suggestion.\(^{52,63}\) At present there is enough evidence to indicate that ROS, especially NO adducts, such as peroxynitrite can stabilize HIF-1α through significant modifications in the PHDs, such as nitrosylation\(^64\) or changes in the redox state of their ferrous iron.\(^65\) Such a mechanism has been suggested to be one of the ways in which stabilization of HIF-1α occurs in cancer.\(^{56,67}\) The role of ROS in the stabilization of HIF-1α in hypoxia has not been completely clarified. However, it is possible that such stabilization might be a two-step process in which a ROS-dependent mechanism that occurs at low [O\(_2\)] is gradually substituted by the lack of function of the PHDs attributable to oxygen starvation in hypoxia. Interestingly, in the presence of NO, which diverts oxygen from the mitochondria and therefore reactivates the PHDs, the stabilizing effect of ROS on HIF-1α appears to be impaired, as has been demonstrated in vascular endothelial cells. In these cells, the activation of AMPK by a ROS-dependent mechanism which occurs at a low [O\(_2\)] is not accompanied by the stabilization of HIF.\(^{59}\)

**Summary**

Hypoxia is likely to play an important role in determining disease progression in a diverse range of pathologies, including vascular disease, cancer, and chronic inflammation. Because of this, it is likely that intervention to prevent or treat hypoxia might have a beneficial effect. Indeed, recent studies using pharmacological inhibitors of HIF hydroxylases reveal a number of interesting therapeutic possibilities.\(^{49}\) The work outlined in the current review has summarized the clear role of components of the mitochondrial electron transport chain in determining cellular oxygen, NO, and ROS availability, and consequently the cellular response to hypoxia. It is not possible to consider oxygen sensing at the cellular level without considering the role of mitochondria. Nitric oxide is a major regulator of mitochondrial respiration and as such may represent a new therapeutic avenue for the manipulation of hypoxic pathways in vivo.

**Note**

It is now generally accepted that the ambient [O\(_2\)] at which most experiments in cells are carried out in vitro are nonphysiological and represent a hypoxic state. Furthermore, "hypoxia" is difficult to define because it is actually a relationship between oxygen supply and demand and thus might vary greatly from one tissue to another. For the purpose of this review we have arbitrarily defined low [O\(_2\)] as that between 1.5% and 3%, and hypoxia as below 1.5% O\(_2\).

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**References**


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