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Review

Control of cytochrome *c* oxidase activity by nitric oxide

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Abstract

Over the past decade it was discovered that, over-and-above multiple regulatory functions, nitric oxide (NO) is responsible for the modulation of cell respiration by inhibiting cytochrome *c* oxidase (CcOX). As assessed at different integration levels (from the purified enzyme in detergent solution to intact cells), CcOX can react with NO following two alternative reaction pathways, both leading to an effective, fully reversible inhibition of respiration. A crucial finding is that the rate of electron flux through the respiratory chain controls the mechanism of inhibition by NO, leading to either a "nitrosyl" or a "nitrite" derivative. The two mechanisms can be discriminated on the basis of the differential photosensitivity of the inhibited state. Of relevance to cell pathophysiology, the pathway involving the nitrite derivative leads to oxidative degradation of NO, thereby protecting the cell from NO toxicity. The aim of this work is to review the information available on these two mechanisms of inhibition of respiration.

Keywords: Free radical; Signaling; Respiration; NO scavenging; Mitochondria; Hemeprotein

1. Introduction

Nitric oxide (NO) is a fundamental second messenger involved in a number of pathophysiological processes, including vasodilatation, platelet aggregation, apoptosis and neurotransmission [1–4]. In addition, NO has been recognized as a potential signaling molecule controlling cell respiration [5]. Among the respiratory complexes affected by NO, cytochrome *c* oxidase (CcOX) is of particular interest. NO inhibition of complex IV is rapid (milliseconds to seconds), potent and reversible [6,7]; moreover, the inhibition efficiency depends on the relative concentrations of O_2 and NO. On the other hand, other respiratory complexes are inhibited more slowly (several minutes to hours) and at higher, non-physiological, NO concentrations (for some complexes in the millimolar range). Owing to the potential pathophysiological significance of the inhibition of respiration by NO, we have investigated the molecular mechanisms of its reaction with CcOX at different integration levels, from the enzyme isolated in detergent solution to respiring cells. This paper aims at reviewing the information available on this issue.

Endogenous NO is enzymatically produced by NO synthase (NOS) [8], an enzyme existing in three distinct NOSs isoforms named, respectively, neuronal (nNOS or type I-NOS), inducible (iNOS or type II-NOS) and endothelial (eNOS or type III-NOS). Interestingly, NO is also produced at the level of the mitochondrion by a mitochondrial NOS (mtNOS), discovered in the middle 1990s based on immunocytochemical evidence [9–12]. Consistently, mitochondria supplemented with L-arginine were able to produce significant amounts of NO [13]. Since its discovery, it has been debated whether this enzyme was representing a fourth NOS isoform: however, in 1997 Ghafourifar and Richter [14] showed that this mitochondrion-bound NOS was functionally active and Ca^{2+}-dependent, as for nNOS and eNOS. Later, Kanai et al. [15] reported that cardiomyocytes from nNOS-knockout mice do not produce NO in the mitochondrion, contrary to wild-type, concluding that the mtNOS is actually a nNOS. More recently this information was confirmed by a biochemical characterization of the isolated enzyme [16]. The existence of a mitochondrial source of NO further suggests a potential bioenergetic role

Abbreviations: CcOX, cytochrome *c* oxidase; NOS, nitric oxide synthase; Hb, hemoglobin

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for NO in the control of energy transduction, in so far as a mitochondrial production of NO may modulate the electron flux levels through the respiratory chain.

Following the 1990 observation by Carr and Ferguson [17] that NO catalytically generated by the *Paracoccus denitrificans* nitrite reductase was able to inhibit the respiration of bovine heart submitochondrial particles, clear-cut evidence showing that NO controls cell respiration was published in 1994 [18–20]. The target enzyme of this inhibitory pattern is CcOX, as supported by the finding that the extent of NO inhibition depends on O_2 concentration ($K_I = 60$ nM at $[O_2] = 30$ μM [19]), showing competition. Since then, a great body of evidence has been accumulated showing that NO inhibits cell respiration by reacting with CcOX at all integration levels, including mitochondria [18–20], cells [21–24] and tissues [25,26], up to in vivo [27,28]. Work on isolated CcOX in detergent solution allowed to assess that at physiological concentrations NO reacts with the metals in the binuclear active site, namely heme a_3 and Cu_B, in different redox states. Reduced heme a_3 binds NO very quickly and with extremely high affinity, the reaction yielding the typical Fe^{2+}-NO nitrosyl-adduct [29,30]:

$$[a_3^{2+}Cu_B^{+}] + NO \rightleftarrows [a_3^{2+} - NO\ Cu_B^{+}] \quad (1)$$

While in the mitochondrial enzyme this reaction is a straight ligand binding, in some prokaryotic oxidases it is associated to the reduction of NO to N_2O [31–33], in a more complex process. A different reaction was shown to occur between NO and oxidized Cu_B, whereby NO is oxidized to nitrite, presumably via the transient formation of a nitrosonium ion (NO^+) [34]:

$$[a_3^{3+}Cu_B^{2+}] + NO \rightarrow [a_3^{3+}Cu_B^{+}NO^{+}] \rightarrow [a_3^{3+}NO_2^{-}Cu_B^{2+}] \quad (2)$$

Both reactions lead to a reversible enzyme inhibition, with formation of either a nitrosyl Fe^{2+} adduct or a nitrite-derivative. These two inhibited states of CcOX display very different light-sensitivity, because only the nitrosyl derivative is photosensitive and can therefore be dissociated by illumination. Taking advantage of this property, a simple experimental protocol was set up in our laboratory to assess the predominance of each pathway as a function of CcOX integration level and specific experimental conditions.

2. The reactions of NO with CcOX intermediates

The view emerging from an extensive investigation of the reactions of NO with the catalytic intermediates of CcOX is that a reaction occurs with either the reduced heme a_3 or the oxidized Cu_B. In Fig. 1, the canonical catalytic intermediates, each containing these two metals in the corresponding redox state, are schematically shown only for the binuclear center. According to a consensus simplified scheme, the catalytic cycle of CcOX can be divided into a reductive and an oxidative limb [35–37]. In

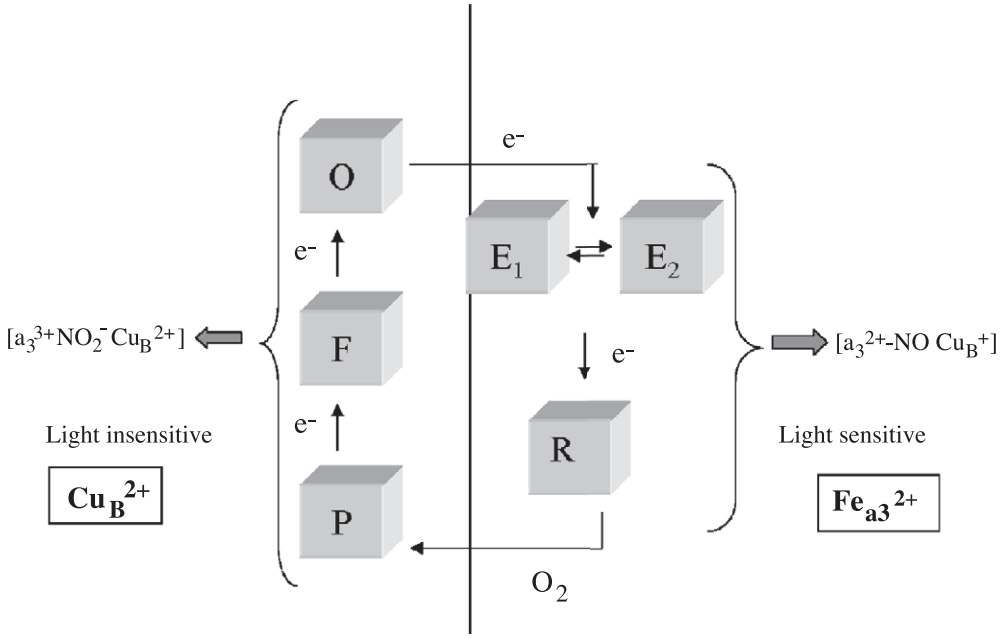

Fig. 1. Reactions of NO with the catalytic intermediates of CcOX. NO reacts with the catalytic intermediates of CcOX (indicated here only at the binuclear center) following two alternative reaction pathways. When reacting at the level of oxidized Cu_B (intermediates O, P and F) NO yields the light-insensitive, nitrite derivative $[a_3^{3+}\ NO_2^{-}Cu_B^{2+}]$. Upon binding to reduced heme a_3 (intermediates E_1 and R), the light-sensitive nitrosyl derivative $[a_3^{2+}$-NO $Cu_B^{+}]$ is accumulated.

the reductive limb, the oxidized active site O accepts two electrons sequentially from Cu_A via cytochrome *a*. This *intra*-molecular electron transfer, eventually yielding the fully reduced site R, proceeds with the formation of half-reduced intermediate E, with the electron residing either on heme a_3 (species E_1) or on Cu_B (species E_2). The rate-determining step in the overall catalytic cycle is the complete reduction of the binuclear site [38,39], which is mandatory for the binding of O_2. Then O_2 is activated and reduced [40] through the much faster oxidative limb of the cycle (microseconds vs. milliseconds) restoring the initial O state via the intermediates P and F [35–37]. Although the fine structure of intermediates P and F is still somewhat debated, it is agreed that both are oxo-ferryl adducts [41].[1]

Intermediates O, P and F contain Cu_B in the oxidized state and, accordingly, they all react with NO following the "nitrite" reaction pathway (see Fig. 1) [34,42–44]; R is characterized by a fully reduced binuclear center, which was shown long ago [45] to bind NO very rapidly, yielding the nitrosyl Fe^{2+} adduct. More difficult was to assess the reactivity of the half-reduced E_1 and E_2 intermediates.

2.1. The reaction of NO with oxidized Cu_B (intermediates O, P and F)

The reaction of NO with the oxidized enzyme (O), investigated by Brudvig et al. in 1980 [46], involves oxidized Cu_B as the reactive metal. However, in those days, CcOX was generally purified in the so-called "resting" or "slow" state, characterized by an active site slowly reacting with exogeneous ligands. The mechanism and the functional relevance of this NO reaction was clarified only in 1997 when Cooper et al. [34], using a so-called "fast/pulsed" preparation of CcOX [47], reported that NO could *rapidly* react with Cu_B in the oxidized enzyme, a reaction prevented in the presence of bound chloride [42]. In the overall process NO is oxidized to nitrite, which then binds to the binuclear site, yielding an inhibited enzyme that upon reduction recovers activity, releasing nitrite in the bulk [44,48]. The reaction of NO with P and F was extensively studied using optical spectroscopy [43,44] and amperometry [44]; both intermediates were found to react at a rate similar to, but smaller than for O ($k \approx 10^4/10^5\ M^{-1}\ s^{-1}$ at 20 °C, [43,44]), yielding the same nitrite-inhibited derivative. Relevant to cell physiology, the reaction of NO with O, P and F yields nitrite, which is then released into the medium; therefore this pathway represents an oxidative degradation mechanism, disposing of toxic NO into harmless nitrite.

[1] By reacting with O_2, the CO-bound two-electron reduced CcOX (so-called mixed-valence-CO adduct) forms P_M, whereas the fully reduced enzyme (R) forms P_R. The reactivity of the short-lived P_R intermediate with NO is essentially unknown so far, therefore throughout this review P stands for P_M.

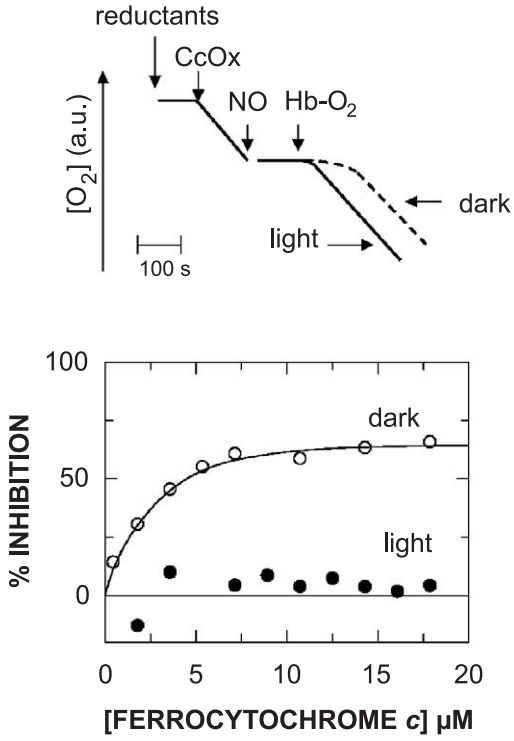

Fig. 2. Light effect on the recovery of CcOX activity. (Top) Schematic representation of the protocol used by Sarti et al. [50]. CcOX-catalyzed O_2 consumption is inhibited upon addition of NO. When free NO is scavenged by addition of excess oxy-Hb, the time course of respiration recovery is followed both in the dark and under illumination. The recovery pattern shown in the figure is characteristic of the light-sensitive "nitrosyl" inhibitory pathway: in the dark, recovery is rate-limited by the slow thermal dissociation of NO from Fe^{2+}, as indicated by the pronounced lag-time; under illumination the recovery rate is accelerated and the lag vanishes. (Bottom) CcOX inhibition assessed as the rate at $t = 50$ s, after removal of free NO by oxy-Hb addition, as measured in the dark and under illumination. At lower cytochrome *c* concentrations, the recovery of activity at 50 s is already complete and not influenced by light, pointing to the accumulation of the "nitrite" CcOX derivative; at higher cytochrome *c* concentrations, the extent of CcOX inhibition is considerably higher in the dark, but vanishes under illumination, because of photodissociation of the light-sensitive nitrosyl Fe^{2+}-NO adduct.

2.2. The reaction of NO with reduced heme a_3 (intermediates R and E_1)

In the early 1960s Gibson and Greenwood [45] characterized from a kinetic viewpoint the reaction of NO with the reduced enzyme R. NO was shown to bind to reduced heme a_3 very quickly ($k = 0.4–1.0 \times 10^8\ M^{-1}\ s^{-1}$ at 20 °C, [45,49]), yielding a tight, photosensitive nitrosyl Fe^{2+} complex. The resulting inhibited derivative recovers activity via dissociation of NO from the binuclear site, a process that occurs slowly in the dark ($k' = 4 \times 10^{-3}\ s^{-1}$ at 20 °C), but that is accelerated by illumination [50]. At first glance, binding of NO to the reduced heme a_3 may seem to account for the efficient inhibition of CcOX, as well as for the competition between NO and O_2, which binds exclusively to the fully reduced state R. However, given that the rate

constants for the binding of O_2 and NO to R are similar, the kinetics of inhibition may be difficult to rationalize, although the data are not necessarily inconsistent with the small inhibition constant ($K_I = 60$ nM at $[O_2] = 30$ μM, [19]). Therefore it was proposed [29,30] that, in contrast to O_2, NO can also bind to a half-reduced binuclear site, E, yielding the heme a_3^{2+}-NO complex. Computer simulations [30] indicated that the additional reacting species E_1 (unique to NO) may be sufficient to account for the kinetic efficiency of the inhibition, as well as the observed competition between O_2 and NO. In support of this hypothesis, consistent experimental evidence was obtained quite recently, by investigating the reduction kinetics of the K354M mutant of *P. denitrificans* CcOX in the presence of NO [51]. Needless to say, it would be desirable to device a procedure to obtain CcOX stabilized in the E state, i.e. with a single-electron in the active site, a task which seems at present unfulfilled.

In summary, the reaction pathway involving NO binding/dissociation from Fe^{2+} of heme a_3 accounts for the functional inhibition and O_2 competition of mitochondrial CcOX, but it should be stressed that NO is temporarily sequestered on the enzyme to be slowly dissociated in the medium as free, reactive radical.

3. Inhibition under turnover conditions

The overall picture emerging from the study of the reactions of NO with the catalytic intermediates of CcOX envisages that two possible derivatives can be formed when the enzyme is exposed to NO, i.e. the Fe^{2+}-nitrosyl [a_3^{2+}-NO] or the nitrite-derivative [a_3^{3+} $NO_2^-Cu_B^{2+}$]. The next relevant question is concerned with the predominance of each of the two pathways under turnover conditions. This issue was addressed by Sarti et al. [50], who designed an experimental protocol based on the well-known photosensitivity of the a_3^{2+}-NO complex [52], to discriminate the formation of this adduct from the light-insensitive nitrite complex. This experimental protocol, initially applied to purified CcOX in detergent solution [50], was recently extended to the enzyme integrated in the membrane, using either mitochondria or intact cells. A scheme of the experiment is shown in Fig. 2 (top panel). Typically, CcOX is allowed to respire in an O_2-electrode vessel until NO is added and respiration is inhibited. After removal of free NO by addition of excess oxy-hemoglobin (Hb), the time course of respiration recovery is followed both in the dark and under illumination. If the "nitrosyl" inhibition pathway is predominant, the recovery of respiration in the dark proceeds at the slow rate characteristic of thermal NO dissociation from Fe^{2+}, and the overall time course is autocatalytic; on the other hand, under illumination, the recovery of respiration is accelerated due to the photochemically induced dissociation of the nitrosyl adduct, and thus the lag time vanishes. In contrast to this pattern, it was shown [50] that if the nitrite-inhibited adduct accumulates, respiration recovery (after scavenging free NO) is promptly restored by reduction of the enzyme, but the O_2 consumption rate is not affected by illumination.

Working with the purified enzyme, it was shown [50] that the predominance of one pathway over the other depends on the concentration of reductants (notably reduced cytochrome *c*) sustaining respiration (Fig. 2, bottom panel). When turnover is sustained by a low concentration of cytochrome c^{2+}, following removal of free NO the recovery is almost immediate (no lag time in the O_2 electrode recording) and light-insensitive, as expected if the nitrite-adduct was the prevailing inhibited state [50]. At higher

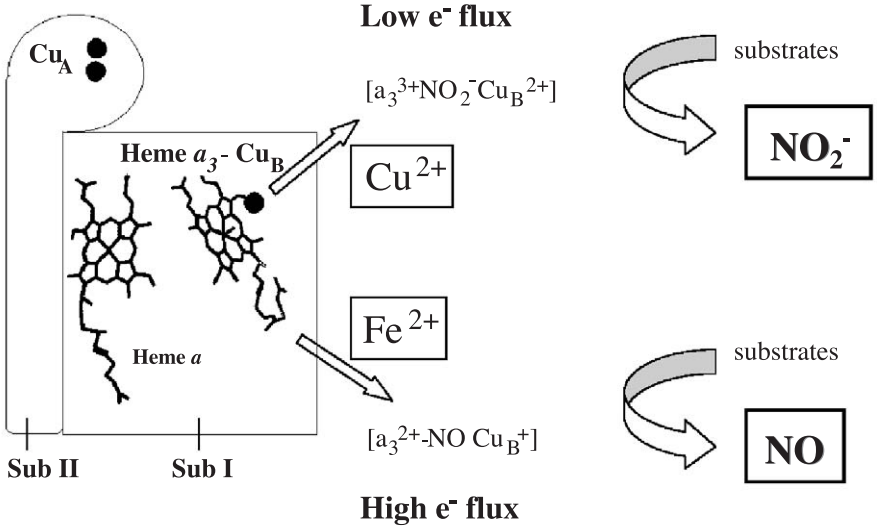

Fig. 3. The two mechanisms of CcOX inhibition by NO. At low electron flux through the respiratory chain, CcOX is inhibited via the "nitrite" pathway, leading to an oxidative degradation of NO under turnover conditions. At higher electron flux, the "nitrosyl" inhibition pathway, associated to NO binding/dissociation at reduced heme a_3, prevails; only the latter pathway accounts for the observed O_2/NO competition.

cytochrome c^{2+} concentrations, however, the autocatalytic recovery in the dark occurs at a rate compatible with the off-rate of NO from the light-sensitive heme a_3^{2+}-NO adduct, and consistently is accelerated by illumination [52]. In summary, at low electron flux nitrite inhibits the oxidase by forming an adduct with the oxidized heme a_3, whereas at higher electron flux the inhibited state is NO-bound to reduced CcOX.

The existence of these two reaction pathways has been substantiated spectroscopically, using soluble CcOX [50]. As schematically depicted in Fig. 3, available evidence suggests that the electron flux through the enzyme controls the predominance of one or the other of the two inhibition mechanisms. During steady-state, a slow rate of electron flux through CcOX increases the overall occupancy of the intermediates having oxidized Cu_B (O, P and F, see Ref. [44]), and thereby the ''nitrite'' inhibition pathway prevails. On the contrary, at higher electron fluxes the probability of forming the nitrosyl Fe^{2+}-NO adduct increases, due to the intrinsically rapid decay of the partially oxidized intermediates [36].

It may be asked whether the existence of these two reaction pathways is somehow artefactually restricted to the purified enzyme in detergent solution. To address this problem, we have investigated the inhibition by NO using isolated mitochondria and cell suspensions. Our results [53] fully confirm that the existence of the two aforementioned inhibitory pathways is not confined to purified CcOX, but is a property of the enzyme integrated in its native membrane.

4. Relevance to cell pathophysiology

NO is a Janus molecule, since depending on its intracellular concentration, it may act as a physiological signaling molecule or as a toxic agent. This is also applicable to its reactions with the respiratory chain, which may be physiologically modulated at relatively low NO mitochondrial fluxes, but severely affected by higher, toxic concentrations. It is known that NO inhibition of mitochondrial respiration has a role in cell death, by either necrotic or apoptotic mechanisms (see Refs. [3,54] for reviews). Moreover, NO toxicity in the extreme is mediated by the reactive peroxinitrite species ($ONOO^-$), formed by the reaction of NO with the superoxide anion; unlike NO, peroxinitrite is indeed causing an irreversible inactivation of respiration. A strict control of intracellular NO may therefore be welcome by the cell.

A physiologically meaningful discussion of the role of NO in the control of respiration cannot ignore the problem of compartmentalization of the NO production, under physiological and pathological conditions. In this perspective, the demonstration of a mtNOS [9–16] acquires special significance and demands some additional considerations. If the density of mtNOS, which is bound to the inner membrane, was comparable to that of the respiratory complexes, then the flux of NO in situ could be sufficient to inhibit a substantial fraction of CcOX under normal turnover conditions. The high solubility of NO in membranes would enhance the probability of encounter with the oxidase and thus of inhibition of respiration, provided that the concentration of L-Arg is sufficient to support a steady flux of NO. This would account for the classical observation that the K_M for O_2 measured in tissues is manifold greater than that of purified CcOX or isolated mitochondria, as lucidly pointed out by Brown [5]. Thus, it may be envisaged that, under normal metabolic conditions, a considerable fraction of oxidase is inhibited, which would have the effect of extending O_2 availability either to cells at different distances from capillaries [16], or within the mitochondrion, allowing O_2 utilization by as many as possible respiratory enzyme complexes, differently distributed in space within the organelle. Within this perspective, the release of NO by dissociation from the a_3^{2+}-NO complex (through one of the inhibitory pathways discussed above) may extend the range of action of NO and thus the effective range of O_2 utilization. On the other hand, the inhibitory pathway involving the formation of the nitrite adduct leads to termination of NO and thus may break propagation. The nitrite-mediated pathway is therefore a mechanism of degradation of NO to harmless nitrite, which is operative in the cell as one of the physiological systems scavenging NO, predominantly in the limit of reduced electron flux.

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