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## Review Article

# The Chemical Interplay between Nitric Oxide and Mitochondrial Cytochrome *c* Oxidase: Reactions, Effectors and Pathophysiology

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Nitric oxide (NO) reacts with Complex I and cytochrome *c* oxidase (CcOX, Complex IV), inducing detrimental or cytoprotective effects. Two alternative reaction pathways (PWs) have been described whereby NO reacts with CcOX, producing either a relatively labile nitrite-bound derivative (CcOX-NO<sub>2</sub><sup>-</sup>, PW1) or a more stable nitrosyl-derivative (CcOX-NO, PW2). The two derivatives are both inhibited, displaying different persistency and O<sub>2</sub> competitiveness. In the mitochondrion, during turnover with O<sub>2</sub>, one pathway prevails over the other one depending on NO, cytochrome *c*<sup>2+</sup> and O<sub>2</sub> concentration. High cytochrome *c*<sup>2+</sup>, and low O<sub>2</sub> proved to be crucial in favoring CcOX nitrosylation, whereas under-*standard* cell-culture conditions formation of the nitrite derivative prevails. All together, these findings suggest that NO can modulate physiologically the mitochondrial respiratory/OXPHOS efficiency, eventually being converted to nitrite by CcOX, without cell detrimental effects. It is worthy to point out that nitrite, far from being a simple oxidation byproduct, represents a source of NO particularly important in view of the NO cell homeostasis, the NO production depends on the NO synthases whose activity is controlled by different stimuli/effectors; relevant to its bioavailability, NO is also produced by recycling cell/body nitrite. Bioenergetic parameters, such as mitochondrial ΔΨ, lactate, and ATP production, have been assayed in several cell lines, in the presence of endogenous or exogenous NO and the evidence collected suggests a crucial interplay between CcOX and NO with important energetic implications.

## 1. Introduction

It is nowadays established that nitrogen monoxide (NO), nitric oxide in the literature, inhibits mitochondrial respiration. The inhibition is induced by the reaction of NO with some of the complexes of the respiratory chain, according to mechanisms studied over more than 20 years. The reaction of NO with Complex III is sluggish [1], whereas the reaction of NO with Complex I and Complex IV, that is, cytochrome *c* oxidase (CcOX), is rapid and to a large extent reversible. Both reactions lead to formation of derivatives responsible of the mitochondrial nitrosative stress observed in different pathophysiological conditions, including main neurodegenerations [2–6]. The functional groups of the mitochondrial complexes reacting with NO include the metals at the catalytic active site of CcOX, namely, the Fe and Cu ions of the

heme *a*<sub>3</sub>-Cu<sub>B</sub> site [7, 8]. The inhibition of Complex I results from the reversible S-nitrosation of Cys39 exposed on the surface of the ND3 subunit [9, 10]. The functional effects on cell respiration depend on the complex targeted by NO and on type of reaction. Inhibition of both Complex I and CcOX is mostly reversible, becoming irreversible, however, depending on duration of the exposure to NO and on its concentration [10, 11]. The onset of NO inhibition on Complex I is slow (minutes [10]), whereas on CcOX is very fast (milliseconds to seconds [12]). In this paper the attention is focused on the interactions between NO and CcOX. The balance between the concentrations of cytochrome *c*<sup>2+</sup> and O<sub>2</sub> proved to be critical in inducing different CcOX inhibition patterns, spanning from a finely tuned *control* to a severe, almost irreversible enzyme inactivation [13]. The interplay between CcOX and NO is based on the inhibition

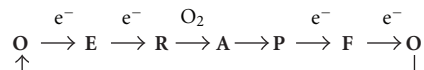
exerted by NO on the enzyme that, in turn, actively controls the NO concentration at the mitochondrial site [14].

The redox active site of CcOX contains one heme  $a_3$  and one  $\text{Cu}_B$  tightly coupled in the so-called binuclear site, where the  $\text{O}_2$  and NO chemistry as well as the reaction with common ligands occur. The active site receives electrons intra-molecularly from the reduced heme  $a$  and  $\text{Cu}_A$ , forming together the electron accepting pole of CcOX, maintained physiologically reduced by cytochrome  $c$ . Also relevant to the reaction of NO with CcOX, the availability in the mitochondrion of reduced cytochrome  $c$  depends on the relative rate at which it is reduced by Complex III and oxidized by  $\text{O}_2$  via CcOX. It is also worth mentioning that the absolute cytochrome  $c$  concentration may vary in different cell lines and tissues [15]. The rate of reaction of CcOX with  $\text{O}_2$  is close to diffusion limited ( $k \approx 1 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$  [16, 17]), whereas the reaction with cytochrome  $c$  is slower,  $k \approx 1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ , the actual rate constant value being dependent on pH and ionic strength [18]. During turnover, the reduction level of the CcOX redox sites, and particularly of the metals in the active site, depends on (i) the actual concentration of reduced cytochrome  $c$  and  $\text{O}_2$  (weighted for their relative  $K_M$  values) at the redox competent sites and (ii) the internal electron transfer rate from the electron accepting pole (heme  $a$ - $\text{Cu}_A$ ), where cytochrome  $c$  reacts, to the active (heme  $a_3$ - $\text{Cu}_B$ ) site, where the  $\text{O}_2$  reaction takes place. At saturating concentration of the physiological substrates, the rate limiting step in the CcOX catalytic cycle is the internal electron transfer [19–21]. Over and above the description of the reaction mechanisms, the aim of this work is to stress the idea that CcOX uses both  $\text{O}_2$  and NO as physiological substrates [5, 14, 22, 23] and to review the experimental evidence pointing to a central role of the NO interplay with CcOX in cell bioenergetics.

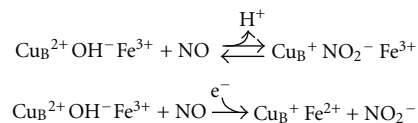
## 2. CcOX Binds Reversibly or Oxidizes NO to Nitrite at the Active Site Where $\text{O}_2$ Binds

In order to better understand the reciprocal interactions between CcOX and NO, it may help summarizing the intermediates populated by CcOX during turnover with physiological substrates. During the catalytic cycle the fully oxidized (**O**) heme  $a_3$ - $\text{Cu}_B$  site accepts a first electron from  $\text{Cu}_A$ /heme  $a$ , leading to formation of a partially, single-electron, reduced (**E**) species; subsequently, a second electron is transferred to the active site, and the fully reduced (**R**) species is formed. Once in the **R** state,  $\text{O}_2$  binds rapidly generating the short-lived (microseconds, at  $20^\circ\text{C}$ ) compound **A**, in which  $\text{O}_2$  is complexed to heme  $a_3^{2+}$  [24]. Electrons are rapidly delivered to bound  $\text{O}_2$ , and Compound **A** converts to a nominal peroxy (**P**) complex with both heme  $a_3$  and  $\text{Cu}_B$  oxidized; actually, the experimental evidence suggests that the O–O (peroxy) bond in this **P** species is already cleaved off, showing heme  $a_3$  in the ferryl ( $\text{Fe}^{4+}=\text{O}$ ) form and a tyrosine residue in a radical state [25, 26]. By accepting a third electron, **P** decays quickly into a canonical ferryl (**F**) intermediate [27], that eventually converts back to the fully oxidized **O** state upon arrival of one last electron from  $\text{Cu}_A$ /heme  $a$ . The sequential steps and the

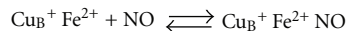
intermediates populated during a single turnover are indicated, starting and ending with the fully oxidized **O** species:



Since first proposed as a unified picture based on experiments carried out using purified CcOX [28], the enzyme adducts formed upon reacting with NO have been spectroscopically identified as a nitrosyl-derivative (heme  $a_3^{2+}$ -NO) or as a nitrite-bound (heme  $a_3^{3+}$ - $\text{NO}_2^-$ ) derivative, or a mixture of these two species, depending on the steady-state fractional accumulation of all the intermediates [29]. It is worthy to notice that the Fe and Cu ions in the active site undergo redox changes only upon reacting in the oxidized state (i.e.,  $\text{Fe}^{3+}$ ,  $\text{Fe}^{4+}$ ,  $\text{Cu}^{2+}$ ) with NO. During the reaction NO is oxidized to  $\text{NO}_2^-$ , that is released in the medium; the whole event is identified as pathway 1 (PW1):



Otherwise, if the active site is partially or fully reduced, an affinity-driven NO binding to these metals takes place; the whole event is identified as pathway 2 (PW2) and occurs without further redox events:



NO is very reactive towards the fully reduced **R** binuclear site. It binds to heme  $a_3^{2+}$  at a rate similar to that of  $\text{O}_2$ , that is,  $k = 0.4 - 1 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$  [16, 17], yielding the high affinity  $\text{Fe}^{2+}$  nitrosyl adduct, whose accumulation is observable directly by spectroscopy or indirectly by NO amperometry [30, 31], when the fully reduced CcOX in detergent solution is mixed with NO. Interestingly, in the presence of NO, all circumstances favoring the electron donation to the catalytic site of CcOX or slowing down its oxidation by  $\text{O}_2$  as during hypoxia (i.e., when the  $[\text{O}_2] \leq K_{M,\text{O}_2}$  of CcOX) proved to favor CcOX nitrosylation [32]. Figure 1 shows schematically how accumulation of the turnover intermediates correlates with the build up of the nitrosylated ( $\text{Cu}_B^+ \text{Fe}^{2+} \text{NO}$ ) or the nitrite-bound ( $\text{Cu}_B^+ \text{NO}_2^- \text{Fe}^{3+}$ ) species.

It is worth mentioning that, contrary to a few bacterial oxidases [34–36], mitochondrial CcOX cannot reduce to  $\text{N}_2\text{O}$  the NO bound at reduced heme  $a_3$  [30]. This implies that the functional recovery of the enzyme after NO binding necessarily lags behind the thermal dissociation of NO from the active site. The dissociation reaction is relatively slow ( $k_{\text{off}} = 3.9 \times 10^{-3} \text{ s}^{-1}$  at  $20^\circ\text{C}$ ) and photosensitive [28]. Photosensitivity has been widely used by Sarti and coworkers to gain insight, through amperometric measurements, into the mechanism of CcOX inhibition by NO in mitochondria or whole cells [37], that is, under conditions unfavorable to spectroscopy. Since the fully reduced binuclear site reacts eagerly with both  $\text{O}_2$  and NO, the inhibition of CcOX via

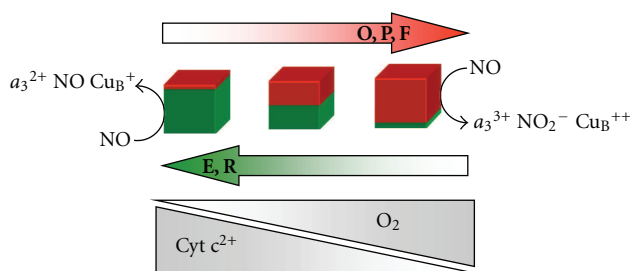


FIGURE 1: Dual-pathway model for the interaction of NO with mitochondrial cytochrome oxidase. The nature of the interaction between NO and CcOX depends on the catalytic intermediates targeted, and these are differently populated at different concentrations of  $O_2$  and reduced cytochrome  $c$ . The oxidized intermediates O, P, F (see text) are overall more populated with increasing  $O_2$  availability, and/or decreasing the concentration of reduced cytochrome  $c$  in the mitochondrion: these intermediates react with NO generating a nitrite-inhibited CcOX. The reduced species E and R (see text) buildup, instead, upon decreasing  $O_2$  and/or increasing the concentration of reduced cytochrome  $c$ : upon reacting with NO, these intermediates generate a heme  $a_3^{2+}$ -NO complex, in competition with oxygen.

formation of a nitrosyl adduct is expected to occur in competition with  $O_2$ , that is, according to PW2. Consistently, the  $O_2$  competition is more clearly observed when the concentration of the reducing substrates favors the reduction of the enzyme [29, 32]. In any case, the inhibition of the nitrosylated CcOX is reverted at the rate of the NO thermal dissociation from reduced heme  $a_3$  [28]. It is worth noticing that, although the NO dissociation process is mechanistically independent of  $O_2$  concentration, bulk  $O_2$  shortens the duration of inhibition by oxidizing free NO in solution, thus hampering NO rebinding to CcOX.

### 3. The Fully- and Half-Reduced Binuclear Site

The ability of the single electron reduced E species to bind NO was investigated using the K354M mutant of the *Paracoccus denitrificans* CcOX [38]. In this mutant the internal electron transfer from the electron accepting pole to the active site is severely impaired, so that the full reduction of the active site and its reaction with  $O_2$  is achieved very slowly, that is, within several minutes. Under these conditions the electron transferred intramolecularly from heme  $a/Cu_A$  resides on either heme  $a_3$  or  $Cu_B$ , and the resulting E species can not react with  $O_2$ . Interestingly, however, E reacts promptly with NO generating the nitrosyl derivative. Thus, one can conclude that, unlike  $O_2$ , NO binds to the binuclear active site even before its complete reduction [12, 31]. Whether the reaction with E plays a role in the mechanism of CcOX inhibition by NO during turnover is still unclear, since it has been also suggested that at steady-state the reaction of NO with E is not required to account for fast inhibition [32, 39]. Regardless of whether the reaction of NO with either E or R is predominant, it seems feasible to conclude that all conditions leading to reduction of the binuclear site in the presence of NO favor nitrosylation of the enzyme.

### 4. The Role of $Cu_B$ in the Reaction with NO

The reaction of NO with  $Cu_B$  in the fully oxidized CcOX to form nitrite was first reported by Brudvig and coworkers in the early 80s [40]. Later on this reaction was reinvestigated by Cooper et al. [41] and Giuffrè et al. [42], using a *pulsed (fast)* preparation of CcOX. The pulsing procedure that *in vitro* consists in preliminary reduction-reoxidation of CcOX [43], removes chloride from the oxidized active site of the enzyme thereby allowing fast reaction with NO [42]; indeed, CcOX is expectedly in the pulsed state *in vivo* where CcOX turnover takes place continuously. During the reaction with the oxidized  $Cu_B$  ( $k = 2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$  at  $20^\circ\text{C}$ ), NO is transiently oxidized to nitrosonium ion ( $NO^+$ ), which is subsequently hydroxylated (or hydrated) to nitrite/nitrous acid.

Thus, after the reaction, the enzyme displays nitrite bound to ferric heme  $a_3$  and is inhibited. The affinity of nitrite for the reduced heme  $a_3$ , however, is much lower than the affinity for the oxidized active site. The intramolecular electron transfer to heme  $a_3$ - $Cu_B$ , therefore, causes the prompt dissociation of nitrite and the subsequent full restoration of activity [29, 44]. Relevant to possible pathological effects of CcOX inhibition by NO, it is worthy to notice that the nitrite dissociation upon reduction of heme  $a_3$  ( $k \sim 6 \times 10^{-2} \text{ s}^{-1}$  at  $\text{pH} = 7.3$ ,  $T = 20^\circ\text{C}$  [29]) is approximately one order of magnitude faster than the NO-dissociation from the nitrosylated site, accounting also for the observed production of nitrite by isolated mitochondria [45, 46].

It has been proposed that nitrite formation could follow an alternative route via reaction with  $O_2$  of the NO bound to the fully reduced CcOX [46]. According to this proposal, a superoxide anion ( $O_2^-$ ) forms by the reaction of  $O_2$  with reduced  $Cu_B$  and reacts with NO bound to reduced heme  $a_3$  to yield peroxynitrite; peroxynitrite is reduced in turn by the enzyme to nitrite, which is finally released in the bulk. The hypothesis, though feasible and intriguing, was not confirmed by independent experiments specifically designed to investigate the kinetics and the products of the reaction of fully reduced nitrosylated CcOX with  $O_2$  [50]. Using myoglobin as an optical probe for free NO, the NO bound to reduced heme  $a_3$  was shown to be displaced by excess  $O_2$  at the low rate of thermal dissociation, to be eventually released in the bulk as such, and not as nitrite [50]. The NO dissociation from the heme iron takes minutes, also when assayed in mitochondria or intact cells, at  $37^\circ\text{C}$  and in the dark, that is, under conditions common *in vivo* in internal organs and tissues. The slow recovery of function of the nitrosylated CcOX is compatible with a more severe state of inhibition characteristic of PW2.

The role of  $Cu_B$  in the CcOX-mediated oxidation of NO to nitrite was also addressed in experiments carried out using the *E. coli* cytochrome *bd*. This oxidase lacks  $Cu_B$  and, consistently, reacts with NO much more slowly ( $k = 1.5 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$  at  $20^\circ\text{C}$ ) than mitochondrial CcOX, without forming nitrite [51]. Interestingly, the NO dissociation from the  $Cu_B$ -lacking cytochrome *bd* oxidase (from *E. coli*) is much faster [52, 53], pointing to a specific property of heme *d* [54] and/or to a role of  $Cu_B$  also in the NO dissociation



TABLE 1: Cytochrome *c* oxidase versus NO—kinetic and thermodynamic parameters.

CcOX intermediate	CcOX adduct formed	$K_I$ (nM)	$k_{on}$ ( $M^{-1} s^{-1}$ ) ( $T = 20^\circ C$ )	$k_{off}$ ( $s^{-1}$ ) ( $T = 20^\circ C$ )	O <sub>2</sub> -competition
<b>E, R</b>	Nitrosylated CcOX-NO	0.2 [32]	$0.4-1 \times 10^8$ [16, 17]	$3.9 \times 10^{-3}$ [28]	yes
<b>O, P, F</b>	Nitrite bound CcOX-NO <sub>2</sub> <sup>-</sup>	20 [32]	$\sim 1 \times 10^5$ ( <b>O, P</b> ) $\sim 1 \times 10^4$ ( <b>F</b> ) [29, 58]	$6.0 \times 10^{-2}$ [29]	no

from the active site. As a matter of fact, this peculiarity was suggested to confer to cytochrome *bd*-expressing bacteria a higher resistance to nitrosative stress [53, 55, 56], a hypothesis supported by *in vitro* studies on *E. coli* deletion mutants of each of the two alternative respiratory oxidases (cytochrome *bd* and cytochrome *bo*<sub>3</sub>) [55].

### 5. Cells Respiring in the Presence of NO and Using Endogenous Substrates

The respiration of cells grown under *standard* conditions, that is, in the presence of (unlimited) O<sub>2</sub> and endogenous reducing substrates, is inhibited by NO but without detectable accumulation of nitrosylated CcOX [37, 57]. As a matter of fact, these standard culture conditions favor the overall accumulation of the CcOX intermediates **P**, **F** and **O** [29, 41, 42, 58]; these are the species responsible for the NO oxidation to nitrite. Consistently, upon rapid and efficient scavenging of bulk NO, respiration is promptly recovered. It is worthy to point out that nitrite, far from being a simple oxidation byproduct, represents a source of NO particularly important in view of the NO cell homeostasis [59–62]. When the oxygen tension decreases in tissues, not only respiration but also the production of NO by nitric oxide synthases (NOSs) is severely impaired, as the NOS uses O<sub>2</sub> as cosubstrate [63]. Anoxia, however, induces tissue acidification, which promotes the reduction of nitrite to NO, compensating for impairment of the NOS-dependent NO production [59, 60, 64]. Consistently, and apparently important for a cardiovascular response, low doses of nitrite ( $\sim 50$  nM) administered to ischemic, heart-arrested mice, early during resuscitation procedures, were shown to significantly improve survival of the treated animals compared to controls [61].

The CcOX NO-inhibition pathway prevailing in mitochondria under given metabolic conditions might be responsible for pathological responses of cells and tissues [57]. Compelling experimental evidence has been collected suggesting that the O<sub>2</sub>-uncompetitive nitrite inhibition pathway (PW1) prevails under conditions of low electron flux through the respiratory chain and high O<sub>2</sub>, whereas the O<sub>2</sub>-competitive nitrosyl pathway (PW2) takes over as the electron flux increases and O<sub>2</sub> concentration decreases [32, 37].

The main features of the two pathways can be summarized as follows:

- (i) both reactions lead to the rapid accumulation of a CcOX inhibited species, characterized by different stability,  $K_I$ , and O<sub>2</sub> competitiveness (Table 1);
- (ii) one pathway prevails over the other one depending on the fractional accumulation of the NO-targeted

CcOX turnover intermediates [28, 29], whose distribution depends in turn on the *in situ* availability of O<sub>2</sub> and reduced cytochrome *c*; the concentration of the latter ultimately depends on its absolute concentration and on the electron flow level through the respiratory chain;

- (iii) PW1 prevails under basal mitochondrial metabolic conditions;
- (iv) PW2 prevails under conditions favoring the accumulation of **E** and **R**, that is, when the concentration of cytochrome *c*<sup>2+</sup> at the CcOX site increases and/or the O<sub>2</sub> tension decreases;
- (v) the accumulation of CcOX-NO or CcOX-NO<sub>2</sub><sup>-</sup> affects differently the mitochondrial bioavailability of NO: the nitrosyl-derivative releases NO in the medium as such, that is, still reactive, whereas the nitrite-derivative releases nitrite to be further oxidized to nitrate, eliminated or rereduced to NO.

The NO concentration level in the cell varies depending on the relative rate of its production, and degradation or scavenging. Unless exogenously supplemented to the cells (NO-donors), the enzymatic endogenous NO production is controlled via the activation/inhibition of the cell NO-synthases. Alternatively, as mentioned above, NO is generated by the protein-bound or free metal ions (Fe<sup>2+</sup>, Cu<sup>+</sup>) catalyzed reduction of NO<sub>2</sub><sup>-</sup>, a reaction that commonly occurs in solution, at acidic pH [59, 60]. The NO bioavailability can be lowered, therefore, by specific cell-permeable NO-synthase inhibitors or by NO scavengers, such as heme-proteins or reduced glutathione [65].

As pointed out by Cooper and Giuliivi [5], when the NOS activity is inhibited, one may expect the O<sub>2</sub> consumption by respiring mitochondria to increase. This event, however, has been often but not always observed [5], probably owing to the activation of alternative NO-releasing systems, such as nitrosoglutathione and S-nitrosated protein thiols, or the NO<sub>2</sub><sup>-</sup> reduction, all active regardless of the presence of NOS inhibitors.

### 6. Effectors and Pathophysiology

Over the years, the enzymatic NO release has been induced in cultured cells, tissues, and organs, either using effectors able to activate cell Ca<sup>2+</sup> fluxes [66], thus stimulating the constitutive NOS, or by enhancing the expression of the inducible isoform of NOS (iNOS) [67]. Morphine is the prototype of a family of drugs used in analgesia and cancer pain treatment [68, 69]. Relevant to the NO chemistry,

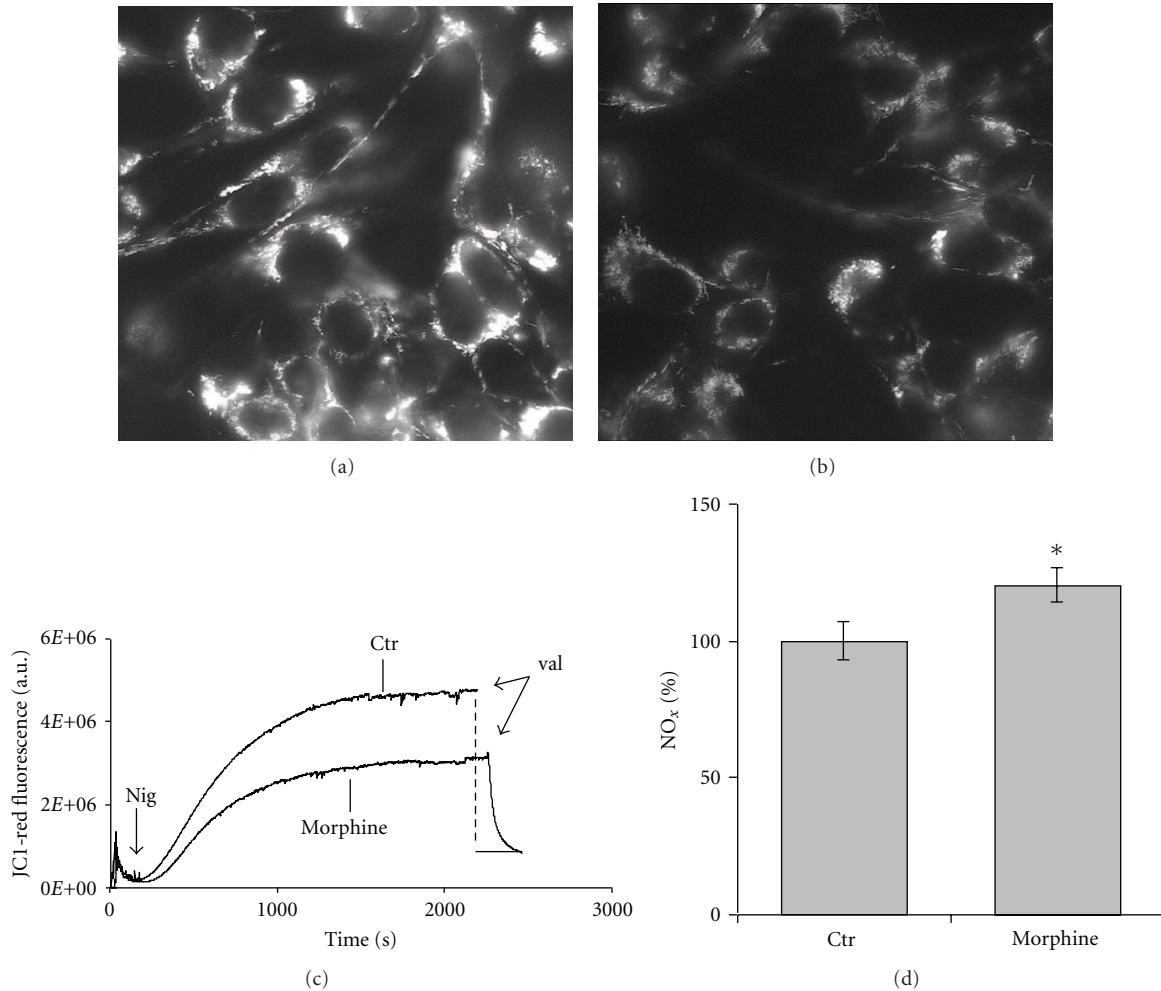


FIGURE 2: Morphine-induced mitochondrial membrane potential and NO<sub>x</sub> changes in Glioma cells. *Fluorescence microscopy*: control cells (a), 20 nM morphine incubated for 24 h (b). Mitochondrial membrane potential ( $\Delta\Psi$ ) was probed using Rhodamine123; the dye is electrophoretically accumulated by the cell mitochondria. *Bulk fluorescence* (c). Control (ctr) versus morphine-treated (morphine) cells, assayed in air-equilibrated medium and in the presence of 2  $\mu$ M ouabain and 0.4  $\mu$ M JC-1; after signal stabilization, 0.6  $\mu$ M nigericin is added and fluorescence changes followed over time. Addition of valinomycin abolishes the membrane potential. Excitation and emission wavelength, 575 nm and 590 nm, respectively. *Nitrite accumulation* (d). The release of NO<sub>x</sub> (nitrite and nitrate) in the medium and during incubation with morphine was assessed spectrophotometrically by the Griess reaction; results expressed as percentage of control cells (ctr). \* $P < 0.05$ . Modified from [33].

morphine activates the opioid and the N-methyl-D-aspartate receptors of neuronal cells, triggering Ca<sup>2+</sup> fluxes and NO release [70, 71]. In 2004, Mastronicola et al. [33] confirmed that the persistence of nanomolar morphine in the cell culture of glioma cells was able to induce the accumulation of nitrite/nitrate in the medium. Interestingly, the cell mitochondria displayed a membrane potential drop off, as probed by a significant decrease of the intramitochondrial JC-1 red-aggregates, whose accumulation requires high mitochondrial  $\Delta\Psi$  values (Figure 2) [72]. Thus, over the same time scale of a cell Ca<sup>2+</sup> transient (seconds to minutes) the NOS activation can affect the mitochondrial potential [33]. More recently, Arese et al. [48] have shown a transient inhibition of the mitochondrial respiratory chain in human adult low calcium

temperature (HaCaT) cells, maintained in a standard culture medium, in the presence of nanomolar (or less) melatonin. After a few hours incubation compatible with a receptor-mediated process [73], and with a timecourse compatible with the circadian melatonin biorhythm, the basal mRNA expression level of the neuronal NOS (nNOS) in the cells was raised by a factor of  $\sim 4$  (Figure 3(a)), returning, thereafter, to basal level [48]. As shown in the same figure, within the same time scale, the authors observed that: (i) the production of nitrite and nitrate (NO<sub>x</sub>) was increased (Figure 3(b)) and (ii) the mitochondrial membrane potential was decreased (Figure 3(c)). Consistently, the ATP<sub>OXPHOS</sub> production was also decreased and an increase of glycolytic ATP and lactate was detected [48]. Taken together, all these findings suggest

that mediated by the melatonin receptors, NO is released and CcOX is reversibly inhibited, with significant bioenergetic consequences. Since cells are not likely facing conditions compatible with the accumulation of CcOX intermediates **E** or **R**, we can infer that inhibition has occurred via PW1. Interestingly, therefore, under physiological conditions, within the limits of a cell culture, a few hours exposure to hormonal-like concentrations of melatonin is able to exert some inhibition on mitochondrial OXPHOS and to raise the  $\text{ATP}_{\text{glycolytic}}/\text{ATP}_{\text{OXPHOS}}$  ratio by a factor of  $\sim 2$  (Figure 3(d)) as expected on the basis of a compensatory physiological Warburg effect [74]. All together these findings suggest that physiological concentrations of melatonin may play a mitochondrial role and interestingly in a circadian context. Indeed, the hypothesis that the melatonin-driven shift towards glycolysis might have a physiological role in the chemistry of the night rest, though attractive, is presently fully speculative, and remains to be investigated.

Based on the effects of melatonin and on the information collected about the NO inhibition of purified CcOX or mitochondria [75, 76], it is also tempting to speculate on how the mitochondrial state can affect the response to NO, particularly under conditions compatible with a limited, and transient raise of NO concentration. It is worthy to consider that isolated state 3 mitochondria proved to be inhibited by NO more effectively than state 4 mitochondria [75, 76]. This suggests that the sensitivity to NO inhibition increases with the electron flux level of the respiratory chain, and particularly with the turnover rate of CcOX; under these conditions the CcOX inhibition is oxygen competitive [32]. In state 3 mitochondria, therefore, and in the presence of suitable amounts of reduced cytochrome *c*, the fractional accumulation of the reduced (**E** and **R**) CcOX species is expected to increase; these species are promptly nitrosylated in the presence of NO. At low turnover rate, as in state 4, the oxidized catalytic intermediates (**O**, **P** and **F**) are expected to be more populated [29], and the NO inhibition predominantly occurs following PW1. Both in state 3 and state 4, if the NO concentration is low (e.g., subnanomolar), the fraction of CcOX inhibited is limited, and the depression of respiration is almost insignificant [77, 78], a finding consistent with an excess capacity of CcOX [79, 80]. When NO persists in the cell environment, as during a prolonged incubation with even low (nM) concentration of NO, and particularly if the turnover rate of CcOX is increased, a substantial inhibition of the respiratory chain is predictable and synthesis of  $\text{ATP}_{\text{OXPHOS}}$  decreases [81]. Under these conditions, glycolysis likely takes place to compensate for ATP loss [82].

## 7. How Does the NO/CcOX Interplay Turn into Pathology

As just mentioned, the transient inhibition of mitochondrial OXPHOS may induce a physiological, compensatory activation of glycolysis [74]. This original observation by Warburg was recently repropounded by Almeida et al. [83], to rationalize the energetic changes of astrocytes and neurons

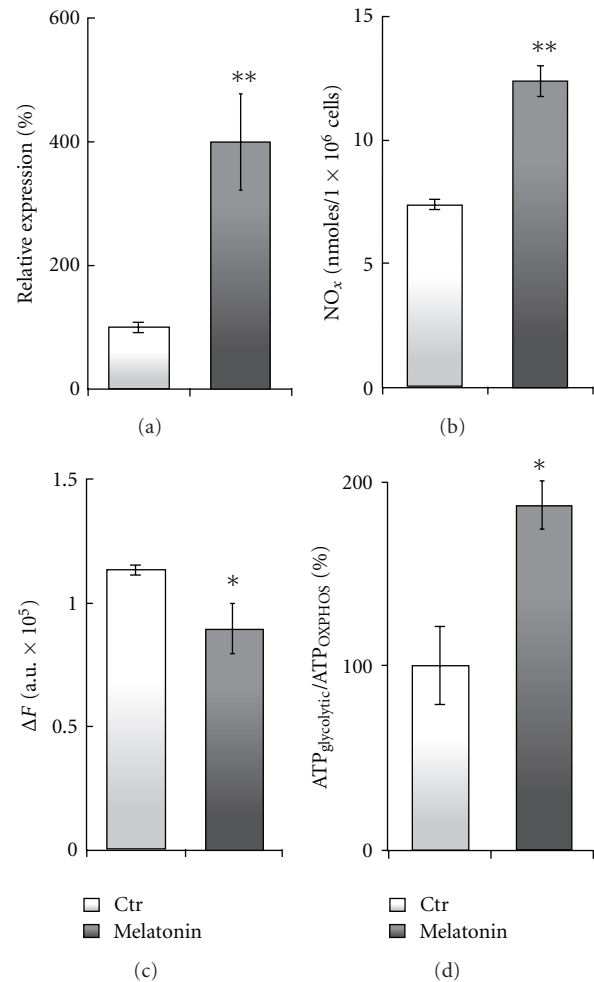


FIGURE 3: Melatonin-induced changes of the nNOS mRNA expression in HaCaT cells: effect on  $\text{NO}_x$  production and mitochondrial membrane potential. (a)—Real-time PCR quantification of nNOS mRNA ( $\beta$ -actin gene used for normalization). (b)—Fluorometric determination of the  $\text{NO}_x$  release in the cell culture medium. (c)—Mitochondrial membrane potential evaluated as the fluorescence difference,  $\Delta F$ , from the maximal (plateau) to the minimal level reached after addition of valinomycin (see also Figure 2(c)). (d)—Contribution of oxidative phosphorylation and glycolysis to ATP production, directly evaluated according to [47]. The  $\text{ATP}_{\text{glycolytic}}/\text{ATP}_{\text{OXPHOS}}$  ratio is indicative of the ability of a given cell line to compensate with glycolysis an OXPHOS impairment (so-called, Warburg effect) [48, 49]. The release of NO induced by melatonin almost doubles the glycolytic contribution to ATP synthesis in HaCaT cells. Cells were incubated with 1 nM melatonin for 6 h. (a), (b):  $**P < 0,01$  versus CTR; (c), (d):  $*P < 0,05$  versus CTR. Modified from [48].

inhibited by NO. In this respect, it is worth considering that neurons, astrocytes, lymphoid, keratinocytes cells, and in general different cell lines may possess a different glycolytic compensatory capacity of coping with OXPHOS NO-inhibition [48, 57, 83]. All the evidence so far collected shows that under standard cell culture conditions, a pulse of NO

leads to the accumulation of the CcOX-NO<sub>2</sub><sup>-</sup> derivative [37], which is able to immediately and fully recover its function, provided that free NO is scavenged in the mitochondrial environment. On the contrary, when CcOX nitrosylation is induced by (artificially) rising the electron flux level at the CcOX site or by allowing the cells to respire towards hypoxia ( $[O_2] \leq K_{M,O_2}$ ), the respiratory chain remains inhibited for longer times at the CcOX site [28, 29, 32, 84]. It is worth recalling that indeed everything else being equal, the functional recovery of CcOX-NO is approximately 10–20 times slower than recovery of CcOX-NO<sub>2</sub><sup>-</sup>. Thus, at least in a first approximation, it is feasible to propose that, compared to conditions promoting the formation of the CcOX-nitrite adduct, conditions favoring CcOX nitrosylation are expectedly more dangerous for cells, since causing a 10–20 times longer inhibition of the mitochondrial respiratory chain. One may indeed speculate that the compensatory glycolytic ATP synthesis might become insufficient, when CcOX is maintained nitrosylated for longer times.

In 2008 Masci et al. [57] characterized the mitochondria NO inhibition pattern of cells collected from patients affected by Ataxia Telangiectasia (AT). This is a multisystemic genetic human disorder characterized by a conjunctival telangiectasia and by a cerebellar degeneration leading to progressive ataxia [85, 86]. The disease is caused by mutations of the AT-mutated gene (ATM), coding for a nuclear 350 kDa protein that controls cell cycle and DNA damage repair [87–89]. AT patients are characterised by a genetic instability and vulnerability to radiation-induced oxidative stress [90–94]. Compared to control cells, AT cells display a defective reactive oxygen species (ROS) scavenging capacity [95, 96], with a decreased bioavailability of reduced glutathione [96].

Relevant to a possible pathological implication of the NO mitochondrial inhibition, AT patients show a bioenergetic deficiency [97]. The mitochondrial functional characterization, and the NO inhibition pattern of lymphoid cells collected from AT patients, proved to be significantly altered. Based on the rate of respiration recovery from inhibition, under otherwise identical conditions of substrates availability (O<sub>2</sub> and reductants), the CcOX in AT cells underwent nitrosylation to a substantially higher extent than in control cells [57]. As expected, based on the higher stability of the nitrosyl-adduct compared to the nitrite-adduct, after NO inhibition and subsequent removal of free NO, recovery of respiration of AT cells is slow, occurring at the rate of the NO displacement from the reduced CcOX active site, whereas control cells recover almost immediately (Figures 4(a) and 4(b)). As a matter of fact the inhibition of AT cells respiration was promptly removed upon shedding light on the cells (photosensitivity of the nitrosyl-adduct!). This peculiarity of AT cells has been correlated to their 1.7 fold higher concentration of mitochondrial cytochrome *c* compared to control cells (Figure 4(c)) [57]. The whole picture is consistent with the hypothesis that in AT cells, showing a lower ATP<sub>glycolytic</sub>/ATP<sub>OXPHOS</sub> ratio compared to control cells (Figure 4(d)), the formation of **E** and **R** and thus CcOX nitrosylation is favored owing to the higher availability of reduced cytochrome *c* [29, 32].

## 8. The Dark Side of the Interplay between NO and CcOX

In conclusion, regardless of the pathway leading to inhibition of CcOX, in the presence of NO, mitochondrial OXPHOS is impaired to some extent. Impairment is due to the slow displacement of NO from the active site or to the involvement of the site in the NO oxidation to nitrite. The evidence so far collected suggests that, if NO remains available in the mitochondrial environment, the mitochondrial membrane potential decreases, and glycolysis begins to contribute significantly to ATP synthesis. Thus, it seems crucial that cells responding to NO pulses are endowed with an efficient glycolytic machinery able to compensate for the decreased aerobic ATP production [82, 83].

Finally, let us consider for the sake of the argument a chronic hypoxia induced by an impaired microcirculation, for instance in the brain. Under these conditions common to many age-related neurodegenerations, one might expect an increased NO release to enhance the blood flow in response to hypoxia. In this already pathological scenario, however, the blood flow and thus O<sub>2</sub> concentration may not increase significantly, owing to the vessel sclerosis; neurons could rather become hypoxic and in the presence of an increased NO concentration. These are the circumstances favouring PW2 (CcOX nitrosylation), even more so if the respiratory chain concentration of reducing substrates is still large enough. Under these conditions and in the absence of a suitable glycolytic compensation, the ATP levels could decrease dramatically, leading to cell death.

## Abbreviations

CcOX:	Cytochrome <i>c</i> oxidase
CcOX-NO:	Nitrosyl cytochrome <i>c</i> oxidase derivative
CcOX-NO <sub>2</sub> <sup>-</sup> :	Nitrite-bound cytochrome <i>c</i> oxidase
PW1:	NO reaction pathway leading to nitrite-bound CcOX
PW2:	NO reaction pathway leading to nitrosyl CcOX
OXPHOS:	Oxidative phosphorylation
ΔΨ:	Membrane electrical potential difference
<b>O</b> :	Fully oxidized CcOX
<b>E</b> :	CcOX with single-electron reduced heme <i>a</i> <sub>3</sub> -Cu <sub>B</sub>
<b>R</b> :	CcOX with fully reduced heme <i>a</i> <sub>3</sub> -Cu <sub>B</sub>
<b>A</b> :	CcOX with ferrous oxygenated heme <i>a</i> <sub>3</sub>
<b>P</b> :	“Peroxy” CcOX intermediate
<b>F</b> :	“Ferryl” CcOX intermediate
NOS:	Nitric oxide synthase
nNOS:	Neuronal NOS
NO <sub>x</sub> :	Nitrite-nitrate
AT:	Ataxia Telangiectasia



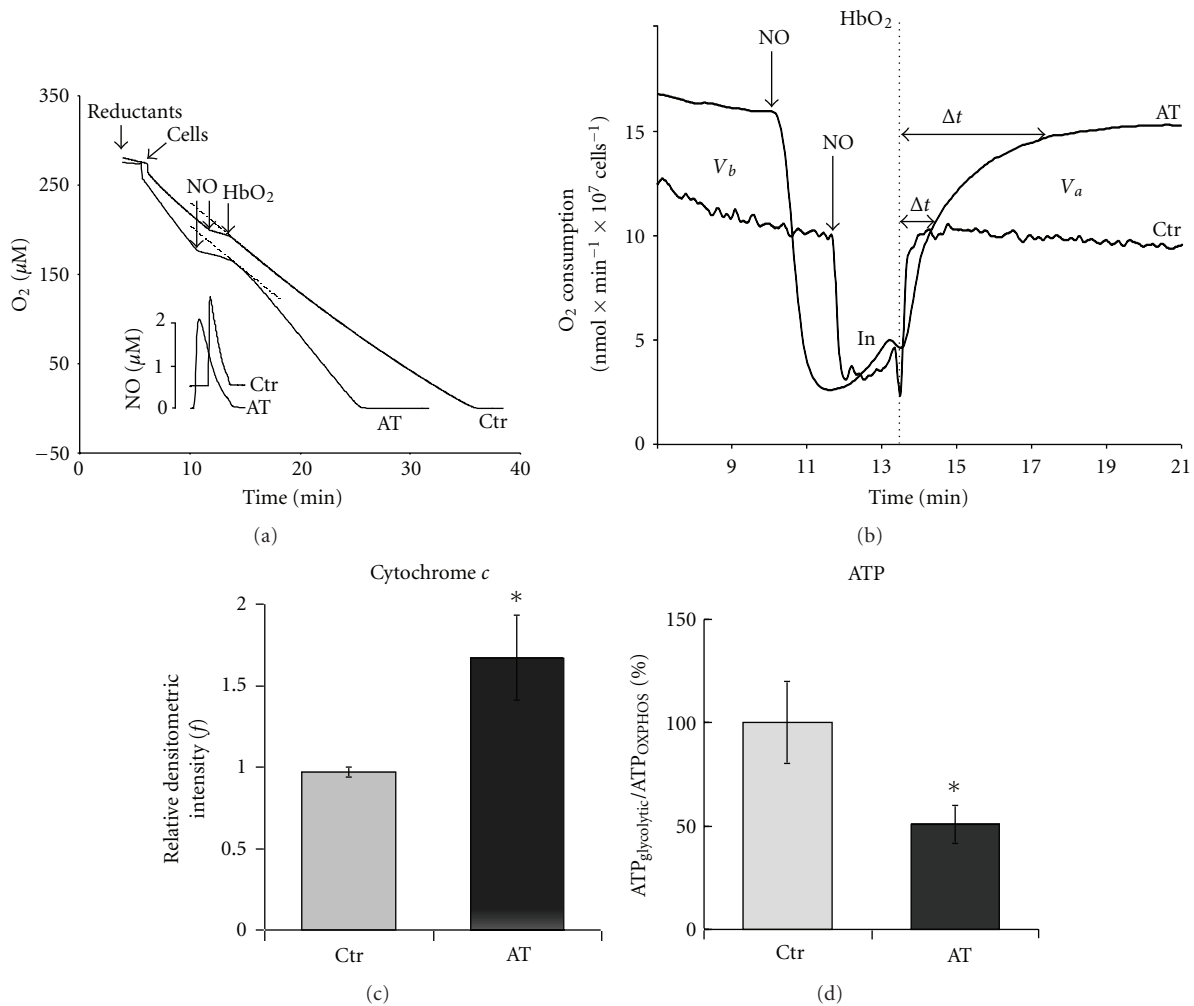


FIGURE 4: Oxygen consumption of Ataxia Telangiectasia (AT) cells: the inhibitory effect of NO. (a)—O<sub>2</sub> consumption profiles of AT and control lymphoblastoid cells, recorded in the dark and in the presence of excess ascorbate and tetramethyl-p-phenylenediamine (TMPD). Inhibition of respiration was induced by adding a single bolus of pure NO gas solution (see lower NO profiles). In order to assess the fraction of residual inhibited CcOX-NO, the instantaneous rate was measured at 45 s after HbO<sub>2</sub> addition. (b)—First derivative plots (integration time  $t = 2$  s). Rate of O<sub>2</sub> consumption before addition of NO ( $V_b$ ), and after addition of oxygenated hemoglobin, HbO<sub>2</sub> ( $V_a$ ), that is, in the absence of free NO. *In*: inhibited state (in the presence of free NO). The  $\Delta t$  value is the time necessary for complete recovery of activity after addition of HbO<sub>2</sub>.  $T = 25$  C. (c)—Cytochrome *c* immunoblot. Cell-lysate (30 μg/well) of AT patients and controls (ctr). (d)—Relative contribution of OXPHOS and glycolysis to ATP production in AT and control cells. Modified from [57].

HaCaT: Human adult low calcium temperature, that is, keratinocytes cell line  
 HbO<sub>2</sub>: Oxygenated haemoglobin  
 State 3 respiration: Induced by ADP, causing a burst of O<sub>2</sub> consumption and ATP synthesis and relaxing into the slower State 4 respiration after ADP consumption.

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