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The effect of nitric oxide on mitochondrial respiration

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ABSTRACT

This article reviews the interactions between nitric oxide (NO) and mitochondrial respiration. Mitochondrial ATP synthesis is responsible for virtually all energy production in mammals, and every other process in living organisms ultimately depends on that energy production. Furthermore, both necrosis and apoptosis, that summarize the main forms of cell death, are intimately linked to mitochondrial integrity.

Endogenous and exogenous •NO inhibits mitochondrial respiration by different well-studied mechanisms and several nitrogen derivatives. Instantaneously, low concentrations of •NO, specifically and reversibly inhibit cytochrome *c* oxidase in competition with oxygen, in several tissues and cells in culture. Higher concentrations of •NO and its derivatives (peroxynitrite, nitrogen dioxide or nitrosothiols) can cause irreversible inhibition of the respiratory chain, uncoupling, permeability transition, and/or cell death. Peroxynitrite can cause opening of the permeability transition pore and opening of this pore causes loss of cytochrome *c*, which in turn might contribute to peroxynitrite-induced inhibition of respiration. Therefore, the inhibition of cytochrome *c* oxidase by •NO may be involved in the physiological and/or pathological regulation of respiration rate, and its affinity for oxygen, which depend on reactive nitrogen species formation, pH, proton motriz force and oxygen supply to tissues.

1. Introduction

Mitochondrial function integrity is key to cell life. Alterations in mitochondrial function have been widely shown to lead to the disruption of cell function, such as tissue or organism disease or even death. Although traditionally regarded as the powerhouse of the cell, the metabolic functions of mitochondria go far beyond bioenergetics. Mitochondria catabolize nutrients for energy, generate macromolecule biosynthetic precursors, compartmentalize metabolites for redox homeostasis maintenance and work as hubs for metabolic waste management. Mitochondrial demands and their complex integration into cell biology outweigh the provision of ATP, a notion which changes our perception of mitochondria and puts organelles in the limelight of cell biology and medicine [1].

Mitochondrial calcium uptake plays a central role in cell signaling and the regulation of mitochondrial function, whereas excessive mitochondrial calcium accumulation has been widely associated with pathological scenarios. Mitochondrial function and dysfunction are thus considered key players in metabolic disease, cancer cell metabolism, neurodegeneration and aging. Mitochondria produce free radical species, possibly also nitric oxide (•NO), and are major targets of oxidative damage. In this sense, several reports have focused on the mechanisms of mitochondrial radical generation and the potential regulatory role of uncoupling proteins, as well as oxidative injury targets [2]. Particularly •NO and reactive •NO species (RNS) have multidimensional effects on mitochondrial function, some of them discussed in this review.

Joseph Priestley discovered both •NO ('nitrous air') and oxygen ('dephlogisticated air') in the early 1770's and performed the reaction between these two gases, producing the soluble brown gas nitrogen dioxide to quantify the amount of oxygen (O_2) in normal air [3,4]. Priestley applied this method to show that mice consumed the O_2 contained in air and thus developed the first accurate measurement of respiration. •NO was subsequently used as a chemical tool, but it was not until the late 1980's that this gas was proven to be produced and utilized by animals, both as a physiological regulator and a cytotoxic agent [5–8].

The paradox of •NO acting as both a physiological regulator and a cytotoxic agent was apparently resolved when •NO was found to react with superoxide to produce peroxynitrite ($ONOO^-$), which is a potent

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oxidant and harmful agent. Therefore, it was suggested that •NO was mostly responsible for the physiological regulation, while peroxynitrite was responsible for the cytotoxic properties [9]. However, this appealing dichotomy may not be as clear-cut as once appeared.

It is well established that •NO causes rapid and reversible inhibition of cytochrome *c* oxidase at low levels, so that •NO is potentially a physiological regulator of respiration. •NO is produced by different isoforms of •NO synthase in different locations within and outside cells, and •NO is consumed (particularly by hemoglobin and myoglobin) in different locations [10]. Interestingly, •NO is a major regulator of O_2 supply via vasodilation of vascular smooth muscle and inhibits the main consumer of O_2 by inhibiting cytochrome *c* oxidase. The fine balance between O_2 delivery to cells and O_2 consumption by the electron transport chain may be due to the gradients of •NO and O_2 which interact in several ways. In this regard, it has been reported that O_2 is a substrate of all •NO synthases; the Km of •NO synthases for O_2 is potentially within the physiological range in the heart. Hemoglobin and myoglobin produce •NO at low O_2 and consume •NO at high O_2, and •NO oxidation of the globins inactivates their ability to transport O_2 (and •NO). Combining the actions of •NO in different tissues and its regulation of blood flow contributes to better substrate delivery and utilization by different tissues, including muscles and myocardium, in basal metabolism or exercise [11]. Therefore, low physiological levels of •NO (1–200 nM •NO) can cause substantial inhibition of respiration, and potentially make tissue respiration highly sensitive to oxygen tension.

In a variety of tissues, organisms and conditions respiration can become very sensitive to the O_2 level, and the competition between •NO and O_2 at cytochrome *c* oxidase might play a role in this sensitivity. Nevertheless, there is no direct evidence for this *in vivo*, the apparent Km of •NO synthase for O_2 is considerably higher than Km of cytochrome *c* oxidase for O_2 (in the absence of •NO), so that at moderately low O_2 levels •NO synthase might be unable to produce sufficient •NO to inhibit cytochrome *c* oxidase [12].

•NO becomes an inhibitor of the enzyme, in competition with O_2. Evidence suggests that at low [O_2] •NO will activate the soluble guanylate cyclase to produce vasodilatation and therefore increase the local supply of O_2. This effect has been suggested to be a significant contributing factor in hypoxic vasodilatation [13]. •NO therefore behaves as a rheostat for respiration and eventually acts to ration the consumption of the O_2 available. Then, elevated •NO from its decreased inactivation by cytochrome *c* oxidase at low [O_2], is a protective mechanism against tissue hypoxia. It could be considered as a useful scenario for the response of tissues to this deleterious condition [14].

Even before •NO was discovered as the responsible agent, it was known that activated macrophages produced a compound which was cytotoxic to other cells by irreversibly inhibiting their mitochondrial respiration [15–17]. •NO and its derivatives peroxynitrite and nitrogen dioxide can indeed irreversibly inhibit mitochondrial respiration; however, a radically different effect of •NO on mitochondrial respiration was reported in 1994 [18]. Very low levels of •NO caused a completely reversible inhibition of mitochondrial respiration at cytochrome *c* oxidase in competition with O_2. In fact, the Ki was estimated to be 270 nM •NO when the oxygen concentration was between 125 and 165 μM O_2, and 60 nM •NO when the oxygen concentration was 18–38 μM O_2, as determined in synaptosomes [19]. This raised the exciting question whether •NO was a physiological regulator of mitochondrial respiration, in fact the only direct regulator of this metabolic pathway known so far. In this context, we will review the effects of •NO on cytochrome *c* oxidase, the impact of •NO derivatives and other radicals on mitochondria, and tisular and cellular •NO measurements *in vivo*, all of which ultimately lead to the modulation of mitochondrial respiration.

2. Cytochrome *c* oxidase

Cytochrome *c* oxidase (cytochrome aa3, complex IV) is the terminal complex of the mitochondrial respiratory chain, responsible for about 90% of O_2 consumption in mammals, and essential for virtually all energy production in cells [20,21]. It is located in the mitochondrial inner membrane, and catalyzes the oxidation of cytochrome c^{2+} to cytochrome c^{3+} and the reduction of O_2 to water, which is coupled to the pumping of protons into the intermembrane space of the mitochondria. This oxidase contains two hemes (cyt a and cyt a3) and two copper centers (CuA and CuB). O_2 binds to the reduced form of a binuclear center consisting of cytochrome a3 (Fe^{2+}) and CuB (Cu^{+}) within the complex, and this constitutes the O_2 binding site and catalytic center of the oxidase. Extensive and relevant data about regulation of cytochrome *c* oxidase, oxidative phosphorylation and metabolic interrelations *in vivo* have been published by Wilson and colleagues, almost since the mid '1960s. These authors' findings and their interpretation have greatly contributed to a broader understanding of the regulation of cellular energy metabolism, particularly the nature, setting and maintenance of metabolic homeostasis. Four critical parameters determine the rate of respiration by cytochrome *c* oxidase under physiological conditions: O_2 pressure, fraction of cytochrome *c* reduced (f_{red}), energy state, and pH [22].

The apparent Km of mitochondrial cytochrome *c* oxidase for O_2 is dependent on the pH of the suspending medium, [ATP]/[ADP][Pi], and the state of reduction of cytochrome *c*. When physiological levels of [ATP]/[ADP][Pi], pH and cytochrome *c* reduction are used in suspensions of isolated mitochondria, oxidative phosphorylation has an O_2 dependence similar to that observed in intact cells [23].

For well-coupled rat liver mitochondria at pH 7.0 and in the presence of ATP, as O_2 concentration decreased, increased cytochrome *c* reduction was found to begin at O_2 concentrations over 20 μM. For mitochondria in the presence of uncoupler, cytochrome *c* reduction started at O_2 concentrations under 1 μM. In other words, O_2 dependence of cytochrome *c* reduction in well coupled mitochondria treated with ATP was tightly associated with the pH of the suspending medium, with cytochrome *c* reduction beginning at higher O_2 concentrations as the pH became more alkaline. O_2 concentration for half-maximal respiratory rates was markedly higher for well coupled mitochondria treated with ATP (around 0.7 μM) than for mitochondria treated with uncoupler (less than 0.1 μM). Therefore, O_2 dependence on mitochondrial oxidative phosphorylation may lead to the conclusion that mitochondria may act as tissue O_2 sensors for the regulation of functions such as local blood flow and electrical activity in the carotid body [24].

Furthermore, a detailed mathematical model of cytochrome *c* oxidase kinetics was recently developed incorporating specific H^+ pumping mechanisms for each e^- transfer step in the catalytic cycle and balancing four H^+ implicated in cytochrome *c* oxidase reaction. This model efficiently describes experimental data available on cytochrome *c* oxidase kinetics with different cytochrome *c* reduced fraction (f_{red}) for different ΔΨ and pH with a physiologically realistic unique parameter set. The model can also make qualitative predictions of the dynamic profiles of O_2 concentration, O_2 consumption rates and cytochrome *c* redox state for varying ΔΨ and pH conditions applied in a respirometer in the presence of artificial e^- donors. This modeling of experimental data on cytochrome *c* oxidase kinetics has demonstrated that cytochrome *c* oxidase flux is maximum under uncoupled conditions and requires very low levels of cytochrome *c* reduced fraction (f_{red}) (Fig. 1). On the other hand, under coupled conditions with or without added ATP, the enzyme turnover was considerably lower even at higher values of f_{red}, indicating the effect of high proton motriz force on the activity of cytochrome *c* oxidase. Experiments have also shown that cytochrome *c* oxidase activity varies significantly at medium pH, with acidic conditions rendering better function than alkaline ones [25].

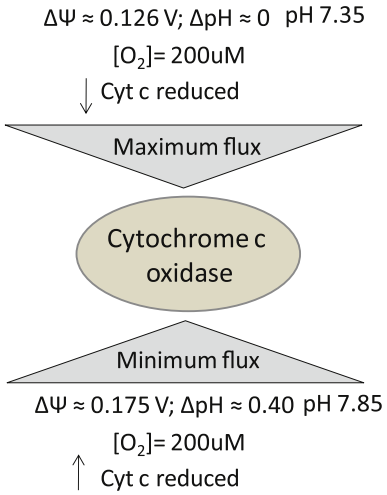

Fig. 1. The apparent Km of O_2 varies considerably and increases from fully reduced to fully oxidized cytochrome *c* (Cyt *c*), both experimental and model simulation results clearly show that the cytochrome *c* oxidase flux is maximum under uncoupled conditions (state 3) and requires very low levels of reduced Cyt *c* (f_{red}), under saturating high Po_2 (analyzed from Pannala et al., [25]).

3. Reversible •NO inhibition of cytochrome c oxidase

•NO binds to the O_2 binding site of cytochrome *c* oxidase, as first reported in 1955 by Wainio [26]. This study found that the addition of •NO to the reduced form of isolated cytochrome *c* oxidase induced a shift in the optical spectrum of cytochromes aa3 similar to the shift induced by carbon monoxide, which suggested that •NO binds to the same site of O_2 on the enzymatic complex, i.e. the reduced form of heme a3. This raised the possibility that •NO could be an inhibitor of cytochrome *c* oxidase in competition with O_2. That •NO is indeed an inhibitor of cytochrome *c* oxidase turnover was first reported in 1994 [18], and extensively studied thereafter [27]. Carr and Ferguson had earlier shown that the product of nitrite and nitrite reductase (presumed to be •NO) inhibited the O_2 consumption of bovine heart submitochondrial particles (inner mitochondrial membranes) [28]. Brown and Cooper [19] showed that 1 μM •NO added to isolated cytochrome *c* oxidase caused an immediate inhibition of O_2 consumption of brain synaptosomes, which was in turn completely reversed when the •NO was broken down. •NO is a potent, rapid and reversible inhibitor of cytochrome *c* oxidase and was apparently competitive with O_2, with a Ki of 270 nM •NO at O_2 concentrations around 145 μM (roughly the arterial level of O_2) and by 60 nM at around 30 μM O_2 (roughly the mean tissue level of O_2), as mentioned in the Introduction section. These levels of •NO are within the measured physiological and pathological range for a number of tissues and conditions, suggesting that •NO inhibition of cytochrome *c* oxidase and the competition with O_2 may occur *in vivo* [19]. A relevant paper by Antunes, Boveris and Cadenas summarizes an *in vivo* biological role of •NO as an inhibitor of citochrome c oxidase and describes various inhibition mechanisms. Competitive inhibition –i.e. resulting from the reversible binding of •NO to reduced cytochrome *c* oxidase– emerges as the single relevant component of cytochrome *c* oxidase inhibition under state 3; in contrast, in state 4, the contribution of uncompetitive inhibition –i.e. resulting from the reaction of oxidized cytochrome *c* oxidase with •NO –constitutes a significant non-majority fraction of inhibition favored by high [O_2]. The principal biological role of the reaction between •NO and oxidized cytochrome *c* oxidase is to consume •NO. Therefore, by consuming •NO, this reaction stimulates, rather than inhibit, respiration [29].

The mathematical model postulated by Pannala and colleagues includes the details of reaction steps in the catalytic cycle in which •NO binds both competitively and non-competitively to inhibit cytochrome *c* oxidase activity. Modeling •NO-mediated inhibition of cytochrome *c* oxidase function proves key to the understanding of both physiological and pathophysiological (e.g., hypoxia) scenarios. The model simulations provide thorough descriptions of experimental data (obtained by Wilson DF and colleagues, see Ref. [22]) for both high and low cytochrome *c* oxidase redox status and reinforce the notion that the inhibitory action of •NO on cytochrome *c* oxidase activity relies on this reduced degree. •NO markedly inhibits cytochrome *c* oxidase activity when cytochrome *c* is highly reduced. Comparable results were obtained from apparent IC50 of •NO inhibition measured at varying levels of O_2 for different redox status for cytochrome *c*, a model which succeeded in accurately fitting experimental data (Fig. 2). These results further prove that •NO inhibition of cytochrome *c* oxidase activity is highly dependent on cytochrome *c* reduction (f_{red}) levels. As a consequence, this cytochrome *c* oxidase model can be considered both mechanistically and thermodynamically constrained and well suited to other components of existing mitochondrial bioenergetics models, with a view to understanding enzyme participation in the control of OxPhos in health and disease states, for example, ischemia-reperfusion injury [25].

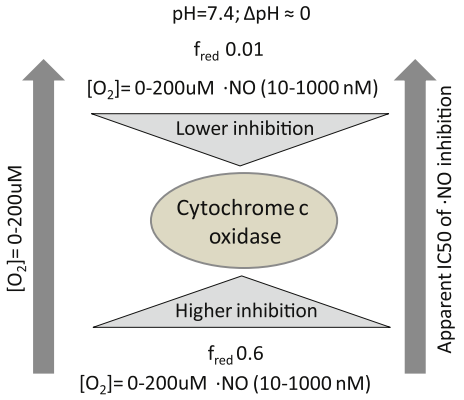

Fig. 2. •NO strongly inhibits the cytochrome *c* oxidase activity when cytochrome *c* is highly reduced (f_{red} 0.6). Apparent IC50 of •NO inhibition increases with varying levels of O_2 for different cytochrome *c* turn over. It is observed that the •NO inhibition of the cytochrome *c* oxidase activity is highly dependent on the level of cytochrome *c* reduction (f_{red}).

Cytochrome *c* oxidase activity of the isolated enzyme, of rat heart submitochondrial particles and brain synaptosomes, as well as the active respiration of mitochondria isolated from rat muscle, liver, heart and brown adipose tissue are all effectively inhibited by 0.05–2 μM •NO [30] and could largely vary (few nM to few uM) depending on several conditions, as mentioned above.

Brown and Borutaite [31] reported that similar levels of •NO reversibly inhibited respiration in isolated heart mitochondria due to the inhibition of cytochrome *c* oxidase, but O_2 consumption was markedly less sensitive to •NO in state 4 than in state 3, probably as a consequence of reduced cytochrome *c* levels, pH values and mitochondrial membrane potential on cytochrome *c* oxidase reducing O_2. Cassina and Radi [32] reported that somewhat higher levels of •NO were required to inhibit O_2 consumption in heart mitochondria but did not actually measure •NO levels. Poderoso and coworkers [33] found that 0.1 μM •NO half-inhibited cytochrome *c* oxidase activity in heart submitochondrial particles, and 0.5 μM •NO half-inhibited O_2 consumption in heart mitochondria at probably high O_2 levels. In turn, Takehara et al. reported that the addition of 1 μM •NO to isolated liver mitochondria caused reversible inhibition of O_2 consumption and ATP synthesis, which was greater at lower O_2 levels [34]. Cleeter et al. showed that a •NO donor caused a reversible inhibition of O_2 consumption in isolated skeletal muscle mitochondria due to the inhibition of cytochrome *c* oxidase [18]. In addition, Lizasoain et al. demonstrated that •NO donors reversibly inhibited the respiration of

submitochondrial particles at cytochrome *c* oxidase level, with an apparent half-inhibition by 2 μM •NO [35]. Finally, Schweizer and Richter [36] found that approximately 1 μM •NO caused a reversible inhibition of O_2 consumption in isolated liver and brain mitochondria, resulting in turn in a reversible depolarization of mitochondrial membrane potential and efflux of mitochondrial calcium, which was greater at lower O_2 concentrations.

In isolated cytochrome *c* oxidase (from beef heart), Torres and colleagues [37] found that •NO binding to the O_2 binding site occurred in a competitive manner, due to •NO binding to a partially reduced form of the cyt a3-CuB binuclear center. Thus, in the presence of O_2 during turnover of cytochrome *c* oxidase, addition of •NO resulted in an optical spectrum identical to that of the oxidase-•NO complex in the absence of O_2. The levels of •NO required for inhibition were somewhat higher than those reported by Brown and Cooper [19], but again the actual levels of •NO were not measured. •NO is known to bind rapidly to the Fe^{2+} of cyt a3 ($k = 0.4–1.0 \times 10^8\ M^{-1}\ s^{-1}$) and also to dissociate surprisingly rapidly ($k = 0.13\ M^{-1}s^{-1}$). In fact, •NO can rapidly inhibit cytochrome *c* oxidase (less than 1 sec) by binding to cyt a3 alone. It has been proposed that •NO preferentially binds to the reduced form of CuB (Cu^+), which then somehow gives •NO a kinetic advantage over O_2 in binding to the Fe^{2+} of cyt a3. Torres and colleagues subsequently suggested and provided evidence that •NO binds to CuB^{2+}, forming Cu^+—•NO^+ [38]. The nitrosonium (•NO^+) may then hydrate to give nitrite, and the Cu^+ may either bind another •NO to form a relatively stable inhibitory complex (Cu^+—•NO), or else the electron may be passed to cyt a3, cyt a or CuA. This binding of •NO to Cu^+ may be the major pathway for rapid inhibition of cytochrome *c* oxidase. However, Giuffre et al. [39] believed that the rapid onset of inhibition may still be explained in terms of fast binding of •NO to the Fe^{2+} of the partially reduced binuclear center. Cassina and Radi [32] further showed that •NO can reduce the fully oxidized cytochrome *c* oxidase in mitochondria (in the presence of antimycin) generating a nitrosyl-(Fe^{2+})-heme a3.

The affinity of •NO for cytochrome *c* oxidase is 5-fold lower when measured by the •NO-induced absorbance shift of isolated cytochrome *c* oxidase than when measured by the inhibition of mitochondrial respiration. This discrepancy might have a number of causes, including the fact that light (which is used to measure the absorbance shift) can dissociate •NO from the oxidase. Indeed, light was found to reverse •NO inhibition of mitochondrial respiration [40].

•NO also potently inhibits cytochrome *c* oxidase activity of bacteria *Paracoccus denitrificans* [28] and *Escherichia coli* [41], as well as that of higher plant mitochondria [42,43]. Some bacterial cytochrome oxidases are homologous to the nitric oxide reductase of denitrifying bacteria, which suggests that cytochrome oxidase may have evolved from denitrifying enzymes. Mitochondrial cytochrome *c* oxidase may in fact have some residual nitric oxide reductase activity [44] as well as •NO oxidase activity, which may contribute to the ability of mitochondria and cells to breakdown •NO. However, twenty years ago, Stubauer et al. reanalyzed the ability of isolated cytochrome *c* oxidase to reduce •NO but found no such activity [45].

Fully oxidized cytochrome *c* oxidase constitutes the key element in the mitochondrial electron transport chain for •NO consumption, as controlled by •NO binding to its binuclear center. •NO consumption by fully oxidized cytochrome *c* oxidase is increased by phospholipid but slowed down by membrane structure and potential when cytochrome *c* oxidase is embedded in the phospholipid bilayer. In the presence of H_2O_2, cytochrome *c* oxidase has been shown to function as a mitochondria-derived •NO peroxidase. Furthermore, a cytochrome *c* oxidase-derived protein radical intermediate has been proven to be induced and involved in the modulation of •NO catabolism [46].

As •NO competes with O_2 at cytochrome *c* oxidase, •NO increases the apparent Km of respiration for O_2 [47], which led Brown and Cooper [19] to suggest that •NO may be a physiological regulator of O_2 sensitivity of respiration in tissues. Koivisto et al. reinvestigated the competition between •NO and O_2 in isolated mitochondria from isolated cortical and outer medullary renal tubules and found that inhibition was consistent with two molecules of •NO competing with one molecule of O_2 [48]. Thus, the IC50 of •NO increased just about in proportion to the square of O_2 tension, and in the presence of •NO the dependence of respiration on O_2 tension had a Hill coefficient of about 2. Since the average level of O_2 in mammalian tissues is about 30 μM, these results emphasize that even at low physiological levels of •NO (1–200 nM •NO) respiration can be inhibited, making mitochondrial respiration highly sensitive to O_2 tension.

The cell membrane is permeable to •NO, and thus •NO (or •NO donors) addition to cells causes rapid and O_2-dependent inhibition of respiration at cytochrome *c* oxidase level in neuronal nerve terminals [49], hepatocytes [50], astrocytes [51], pancreatic β-cells [52], and ascites tumor cells [53]. This respiratory inhibition is largely though not completely reversed when •NO is removed, which hints at an irreversible component in this inhibitory effect. The inhibition of respiration by •NO results in a reversible and O_2-dependent decrease in mitochondrial membrane potential, an increase in cytosolic calcium (probably due to efflux of mitochondrial calcium) in several cellular types mentioned above, and a decrease in cellular ATP in ascites tumor cells [53]. •NO-induced respiratory inhibition is also accompanied by insulin release by β-cells and intact pancreatic islets [52] and glutamate release by nerve terminals [49]. •NO donors also inhibit cellular O_2 consumption and reduce the ATP level of lung alveolar type II cells [54].

However, the above findings that addition of •NO to *in vitro* systems causes inhibition of respiration at cytochrome *c* oxidase do not provide conclusive evidence that •NO is a physiological regulator or pathological inhibitor of mitochondrial respiration. For this purpose, it is necessary to determine whether •NO produced endogenously by •NO synthase (NOS) in cells causes reversible inhibition of cellular respiration.

4. Tissue •NO measurements *in vivo*

The measurements of •NO levels *in vivo* have been previously described and performed by different techniques like Malinski's •NO-selective electrode, microsensors for •NO and carbon fiber microelectrodes in living tissues [55]. Abundant literature has presented detailed studies of •NO concentration and its changes in dynamics *in vivo*. Relevant evidence demonstrates heterogeneous •NO concentration dynamics in the hippocampal subregions, functionally dependent on the stimulation of the NMDA subtype of glutamate receptors, which respond differently in terms of •NO production. In the CA1 pyramidal cell layer, •NO concentration reached the highest levels (typically ≈250 nM) but, even under conditions of continuous NMDA stimulation, •NO rose only transiently [56]. By selective microelectrodes coupled with rapid electrochemical recording techniques, •NO endogenously produced in hippocampus *in vivo* was measured for the first time, following activation of ionotropic glutamate receptors [57]. Using microelectrodes inserted into the living brain of anesthetized F344 rats, glutamate-induced •NO concentration was observed to decrease in the hippocampus, striatum, and cerebral cortex as animals age increased, suggesting that the impairment in the glutamate:nNOS pathway represents a critical functional event in cognitive decline during aging [58].

•NO concentration dynamics *in vivo* in response to activation of ionotropic glutamate receptors were analyzed by two glutamatergic agonists, L-glutamate (the endogenous agonist) and NMDA (the specific and synthetic agonist of NMDA receptor), as well as their modulation with specific antagonists. Both agonists were able to produce robust and transient elevations of •NO levels, with peak concentration reaching the low micromolar range based on the precalibration of the microelectrodes with a saturated •NO gas solution. By means of direct and real time measurements of •NO production, it was shown that the

production of this radical, due to the activation of the nNOS, may be modulated by 7-nitroindazole (7-NI) administrated intraperitoneally and that •NO production elicited by L-glutamate is the result of an integrated activation of ionotropic glutamate receptors, both NMDA and α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid (AMPA) receptor [59].

Apart from brain, basal •NO production rates and changes in •NO production were measured in response to ATP with temporal and spatial resolution inprimary human endothelial cells within the microvessel network by means of fluorescence indicators.The perfused microfluidic device provides an *in vitro* model that allows a close simulation of the *in vivo* microvascular geometries and shear flow conditions. •NO production can be measured in these conditions, as a useful tool to investigate how pathologically altered blood components from patient samples affect human endothelial cells and provide insight into clinical issues [60].

Moreover, the use of NOS inhibitors *in vivo* has shown that inhibiting basal •NO production causes marked tissue stimulation and whole-body O_2 consumption, consistent with a basal inhibition of tissue respiration by •NO. However, this stimulation might be mediated by cGMP or another mechanism rather than by cytochrome *c* oxidase inhibition. Therefore, further *in vivo* experiments are required to determine whether •NO is indeed a direct physiological regulator of oxidative phosphorylation.

5. •NO produced *in vivo* by cells and tissues and effects on mitochondrial respiration

Research into whether •NO can regulate *in vivo* cellular and tissue respiration is crucial but complicated for several reasons such as: (a) •NO can react with cellular components to produce peroxynitrite, nitrosothiols and other derivatives, which may affect respiration generally by an irreversible inhibition; (b) •NO is a potent vasodilator which increases O_2 supply; (c) •NO may affect ATP consumption indirectly modulating cellular O_2 consumption; (d) •NO can affect muscle contraction by cGMP-dependent mechanisms; and (e) •NO reacts rapidly with hemoglobin and myoglobin, as oxygenated myoglobin functions as a •NO scavenger to prevent •NO-mediated inhibition of mitochondrial respiration and hence contributes to higher rates of oxidative phosphorylation [61].

A variety of cells, including macrophages, astrocytes, hepatocytes and myocytes, can be induced to express the inducible form of NOS (iNOS) by cytokines, endotoxins and/or oxidative stress, which leads to the production of a sustained high level of •NO [62,63]. Primary cultures of astrocytes isolated from brain were activated to express iNOS by interferon-γ and endotoxin and were found to produce up to 1 μM •NO. This endogenous •NO was observed to cause a potent inhibition of cellular respiration at cytochrome *c* oxidase, which was rapidly reversed in part by either removing this radical with oxyhemoglobin or inhibiting the NOS enzyme [64]. Furthermore, Venema has reported that primary cultures of endothelial cells which express the constitutive endothelial form of NOS (eNOS) released a brief pulse of •NO upon stimulation with bradykinin or ATP, causing reversible inhibition of cellular respiration [65]. Miles et al. found that inhibition of NOS in lung alveolar type II cells caused an increase in cellular O_2 consumption and ATP concentrations, while a •NO donor caused the opposite effect, suggesting that constitutive •NO production can inhibit respiration [66]. Shen and coworkers found that the addition of endothelial agonists bradykinin or carbachol to slices of skeletal muscle *in vitro* caused •NO-dependent inhibition of O_2 consumption. A cell-permeable form of cGMP (8-Bromo-cGMP) also caused O_2 consumption inhibition of muscle slices, which indicates that this effect is at least partly mediated by cGMP [67]. However, in the presence of mitochondrial uncoupler 2,4 dinitrophenol, 8-Bromo-cGMP failed to inhibit respiration while bradykin in still succeeded, which suggests that at least part of the inhibition was due to a direct effect of •NO on the respiratory chain. Poderoso et al. used isolated perfused rat hearts and showed that bradykinin caused a 30–40% decrease in O_2 consumption, associated with an increase in •NO concentration in the effluent and vasodilation of the coronary vasculature, but •NO significant decrease in left ventricular pressure [68]. Worth pointing out, these effects were blocked by NOS inhibition through the use of N-monomethyl-L-arginine. These results suggest that bradykinin-evoked •NO release from the endothelium can inhibit myocyte O_2 consumption. However, as once again reversibility, O_2 and cGMP dependence of the inhibition were not measured, inhibition mechanisms remain unclear.

In *in vivo* studies, Shen et al. found that NOS inhibitor NMA, N-nitro-L-arginine in conscious dogs caused a rapid and sustained 25% increase in whole-body O_2 consumption (estimated from cardiac output and O_2 extraction), even though O_2 availability for tissues was decreased due to hemodynamic changes [67]. In turn, barbiturate (pentobarbital)-anesthetized dogs did not show the NMA-induced stimulation of O_2 consumption. The same authors found that NMA also increased O_2 consumption in dogs hind limb skeletal muscle and kidneys by 58% *in vivo*, although sodium reabsorption was decreased, which suggests that kidney respiration was inhibited directly by endogenous •NO. King et al. found that NOS inhibition caused a 40% increase in O_2 consumption in hind limb skeletal muscle of anesthetized dogs despite a decrease in blood flow [69]. Also, NMA caused a 28% increase in heart O_2 consumption in exercising dogs.

Altogether, these results are consistent with the interpretation that basal constitutive •NO release by capillary endothelium or by NOS within muscle cells inhibits tissue respiration. However, mediation of this inhibition by cGMP and/or ATP consumption cannot be ruled out, as cGMP can weakly inhibit muscle O_2 consumption *in vitro* and possibly *in vivo* in rabbit hearts [70]. At variance with the concept that cGMP can inhibit muscle and heart O_2 consumption, it has been recently reported that cGMP and •NO donors can stimulate the oxidation of glucose, pyruvate, palmitate and leucine in skeletal muscle via a cGMP-dependent mechanism [71]. This stimulation was suggested to be involved in the physiological stimulation of glucose oxidation by muscle contraction, and it was speculated that cGMP-dependent protein kinases might stimulate respiration by phosphorylating uncoupling proteins in the mitochondria. However, the mechanisms of *in vitro* cGMP-dependent stimulation remain unclear, with possibilities including increased perfusion of the muscle or stimulation of ATP utilizing processes in the muscle. More work is clearly needed to untangle the multiple effects of •NO/cGMP on respiration.

6. Effects of •NO in tissue mitochondria (apart from cytochrome *c* oxidase inhibition)

Poderoso et al. reported that •NO inhibited the cytochrome bc1 complex (complex III) in submitochondrial particles. This inhibition was partially reversible and occurred at •NO concentrations somewhat higher than those inhibitingcytochrome c oxidase [33]. However, the inhibition observed may be due to the S-nitrosoglutathione/dithiothreitol system used to generate •NO, or to peroxynitrite generated from •NO and superoxide in this system. Poderoso et al. [72] also demonstrated that •NO can react directly with ubiquinol-2, an analog of endogenous mitochondrial electron transport carrier ubiquinol-10, which might in part mediate •NO inhibition of complex III activity or •NO induction of mitochondrial superoxide production.

Welter *et al.* [73] treated purified complex III, succinate-ubiquinone reductase complex II or complexes II and III together as a supercomplex with 2 mM •NO for 1 min in strictly anaerobic conditions; these very high levels of •NO in the absence of O_2 resulted in 40% inhibition of the activity of complex III alone but no inhibition on the supercomplex. It may be inferred that high levels of •NO induce a reversible change in the structure of Rieske iron-sulfur center protein, without loss of the iron, but that complex III is protected from •NO when in a supercomplex with complex II. Also, high levels of •NO in the absence of O_2 resulted in

the total inhibition of complex II activity. This finding suggests that •NO itself has little effect on complex III, although very high concentrations of •NO can irreversibly inhibit complex II by destroying iron-sulfur centers.

Keilin, the discoverer of cytochromes [74], found that •NO binds to cytochrome *c*. •NO reversibly binds to oxidized (ferri-) cytochrome *c* with a low rate of binding ($10^{-3}\,M^{-1}.s^{-1}$) and a high dissociation constant (20 μM), being only significant at very high levels of •NO. •NO also slowly reacts with reduced (ferro-) cytochrome *c* ($200\,M^{-1}\,s^{-1}$) to form ferri-cytochrome c and the nitroxyl ion (•NO−), which may further react with O_2 to produce peroxynitrite. This might be a significant source of peroxynitrite independent of superoxide production, and the nitroxyl ion itself can be cytotoxic.

Creatine kinase, which buffers cellular ATP levels, is slowly inhibited by •NO, probably via S-nitrosylation, while inhibition is reversed by dithiothreitol (DTT) [75]. In contrast, peroxynitrite rapidly and potently inhibits creatine kinase via oxidation of protein thiols, an inhibition not reversed by glutathione.

7. Irreversible inhibition of mitochondrial respiration by •NO

In addition to its roles in normal physiology, it is important to appreciate that •NO also has pathophysiological actions. •NO reacts with various O_2 species in the cell to form highly reactive molecules that damage cellular components through various mechanisms.

Cells exposed to •NO (or •NO-producing cells) show immediate but reversible inhibition of respiration at cytochrome *c* oxidase. However, after several hours of exposure to •NO, irreversible inhibition arises probably due to conversion of •NO to reactive nitrogen species (RNS) which inhibit respiration at multiple sites. One of the most rapid effects is the inactivation of complex I, possibly due to S-nitrosation of the complex [76], followed by inhibition of aconitase and complex II, possibly due to the removal of iron from iron-sulfur centers [77] under conditions where peroxynitrite may be formed. The mechanism(s) by which •NO and RNS inhibit complex I remain unclear. S-nitrosothiols, S-nitrosoglutathione and S-nitrosoacetylpenicillamine can all rapidly inactivate complex I when added to isolated mitochondria, even in conditions when little or no •NO is released. This inhibition is reversed by light or reduced thiols (glutathione or DTT), which suggests that the inactivation is mediated by transnitrosation. However, the inhibition of complex I induced by peroxynitrite (which is a poor nitrosating agent) is also partly reversed by light and reduced thiols, which indicates that mechanisms other than nitrosation might be involved. It has been suggested that peroxynitrite inhibits complex I by tyrosine nitration [78] and •NO-induced inhibition of complex I in isolated mitochondria was prevented by peroxynitrite scavengers. An alternative (but not exclusive) target might be one or more iron-sulfur centers in complex I. High concentrations of •NO can destroy iron-sulfur centers by binding and displacing iron, and damage must start with •NO binding/reacting with iron and/or cysteine residues which bind iron; this initial phase of inhibition might be reversible by light or reduced thiols. •NO-induced thiol depletion seems to precede inhibition of complex I, which is in turn reversed by reduced thiols. Cassina and Radi [32] found that treatment of rat heart mitochondria with 5 μM •NO in the presence of O_2 resulted in an inhibition of respiration with succinate which was only partially reversible by removing •NO, whereas respiration on NADH-linked substrates (glutamate and malate) was almost completely recovered when •NO was removed. The irreversible inhibition of respiration with succinate by high levels of •NO might be due to •NO-induced destruction of complex II or to peroxynitrite (or other oxides of •NO) formation which may then inhibit complex II. Whatever the mechanisms involved, •NO inhibition of complex I is key to cell dysfunction and death.

8. Peroxynitrite-mediated mitochondrial respiration inhibition

•NO reacts rapidly with superoxide ($\cdot O_2^-$) to produce peroxynitrite ($ONOO^-$), which may act as an oxidant itself, isomerize to nitrate, or protonate and dissociate to give nitrogen dioxide ($\bullet NO_2$) and hydroxyl radical (·OH), all of them strong oxidants [79,80]. Peroxynitrite reacts with protein and non-protein thiols, tyrosine residues, unsaturated fatty acids, DNA, •NO and a variety of other molecules. Addition of peroxynitrite to mitochondria causes extensive protein modification and cross-linking and lipid peroxidation [81], which constitute multiple complex effects.

Peroxynitrite can inhibit complex I, complex II/III, cytochrome oxidase (complex IV), ATP synthase, aconitase, manganese-containing superoxide dismutase (MnSOD), creatine kinase and probably many other proteins [78]. Peroxynitrite is a strong oxidant and can also cause DNA damage, induce lipid peroxidation and increase mitochondrial proton (and other ion) permeability, probably by lipid peroxidation or thiol cross-linking.

Addition of peroxynitrite to mitochondria also causes irreversible inhibition of respiration at a number of sites. It should be pointed out, however, that quite high levels of peroxynitrite (100–500 μM) are required to trigger this inhibition, and most of the peroxynitrite present spontaneously decomposes to nitrate with a half-life of about 1 sec. Also, this inhibition can be prevented by low levels of thiols (50% protection by 10 μM reduced glutathione) or high levels of sugars (50% protection by 8 mM glucose), due to peroxynitrite reaction with these substances, and may also be suppressed by many common buffer components such as HEPES. A sustained release of peroxynitrite (rather than a bolus addition) can be obtained either by adding an •NO donor together with a superoxide source such as xanthine oxidase and hypoxanthine [82], or by adding SIN-1, which is a donor of both •NO and superoxide. This poses disadvantages, however, as peroxynitrite can react with both •NO and superoxide to produce unknown products.

Cassina and Radi showed that addition of peroxynitrite to isolated mitochondria caused inhibition of complex V (the ATP synthetase, IC50 0.6 mM), complex II (succinate dehydrogenase, IC50 0.9 mM) and complex I (NADH-ubiquinone oxidoreductase, IC50 1.5 mM), but relatively little inhibition of complex IV (cytochrome oxidase, IC50 > 2 mM) [32]. Peroxynitrite 0.5 mM caused 76% inhibition of succinate-dependent respiration and 65% inhibition of glutamate/malate-dependent respiration, while 1 mM peroxynitrite caused •NO inhibition of ascorbate/TMPD (N,N,N′,N'-tetramethyl-p-phenylenediamine)-dependent respiration [83]. Although these are very high levels of peroxynitrite, the infusion of 1.3 mM peroxynitrite over a 10 min period (resulting in a low micromolar steady-state level of peroxynitrite) caused the same level of inhibition as a bolus addition of this reagent. In similar experiments, Lizasoian and coworkers found that 200 μM peroxynitrite irreversibly half-inhibited mitochondrial respiration on NADH or succinate, but caused little inhibition of respiration on cytochrome *c* oxidase substrates (ascorbate and TMPD). The mechanisms of peroxynitrite-induced inhibition of complexes I, II, and V are still unclear, as hydroxyl radical scavengers do not prevent inhibition, while peroxynitrite anion scavengers such as thiols, urate and sugars do, which suggests that peroxynitrite itself causes the damage. The mechanisms might also involve the modification of protein thiols or the destruction of iron-sulfur centers in complexes I and II. Peroxynitrite-induced inhibition of succinate dehydrogenase and fumarate reductase in *Trypanosoma cruzi* was reversed by dithiothreitol, which indicates that inhibition was due to nitrosylation of a critical thiol in the protein. Peroxynitrite also inactivates thiol-containing enzymes of *Trypanosoma cruzi* energetic metabolism and inhibits cell respiration [84]. Addition of 1 mM peroxynitrite to isolated brain mitochondria caused partial inhibition of complex II–III activity but no inhibition of complex I or complex IV activity. Brookes et al. [85] reported that this inhibition was also accompanied by increased proton leak causing mitochondrial uncoupling, which was prevented by Trolox, a lipid soluble antioxidant.

Peroxynitrite (but not •NO) can inhibit isolated cytosolic and mitochondrial aconitase, an essential component of the Krebs cycle, which may cause the inhibition of mitochondrial respiration depending on which substrates are being oxidized and whether aconitase limits respiration [86]. The inhibition of isolated aconitase can be reversed by adding iron and thiols such as dithiothreitol or glutathione and appears to result from the removal of the α-Fe of the iron-sulfur complex. Only very high levels of •NO (> 100 μM •NO in the absence of O_2) cause small reversible inhibition, but nitrosothiols cause direct inhibition of aconitase not mediated by •NO [87]. However, the relative importance of •NO and peroxynitrite in inhibiting aconitase remains controversial.

Peroxynitrite can cause the opening of the permeability transition pore (PTP), which causes loss of cytochrome *c* [88] and might in turn contribute to peroxynitrite-induced inhibition of respiration. Pore opening is also likely to uncouple the mitochondria by increasing proton permeability. Peroxynitrite also causes rapid oxidation of mitochondrial NAD(P)H by unknown mechanisms (possibly direct oxidation), followed by hydrolysis of NAD by a cyclosporin-inhibitable mechanism. NAD(P)H oxidation might be associated to the fact that peroxynitrite also rapidly oxidizes mitochondrial glutathione.

There is some controversy concerning inhibition of complex II and complex III by peroxynitrite: some authors have shown that peroxynitrite (and •NO) inhibit complex II with little or no effect on complex III, whereas others find complex III inhibited and complex II unaffected by peroxynitrite [88,89]. In addition, peroxynitrite has relatively little effect on the Vmax of cytochrome *c* oxidase when added to mitochondria at levels that inhibit the other complexes. However, it does have various damaging effects on isolated cytochrome *c* oxidase, including particularly increasing the Km for O_2. High concentrations (> 1 μM) of •NO (possibly via $•NO_2$ or N_2O_3) can also induce an irreversible rise in Km for O_2 both in isolated cytochrome *c* oxidase or in cells treated with •NO.

9. •NO-induced ROS, RNS and mitochondrial permeability transition

Apart from inhibiting respiration, •NO has two other effects on mitochondria relevant to the induction of cell death: (i) induction of reactive O_2 species (ROS) and RNS production from mitochondria, and (ii) induction of mitochondrial permeability transition (MPT) by RNS.

The mitochondrial respiratory chain can produce superoxide, which dismutates to hydrogen peroxide, while inhibition of the chain may enhance the production of these ROS. At moderate levels, •NO can acutely increase $·O_2^-$ and H_2O_2 production by inhibiting mitochondrial respiration, while at higher levels it inhibits H_2O_2 production by scavenging the precursor superoxide, resulting in peroxynitrite production. •NO may also apparently react with ubiquinol (QH_2) to produce $•NO^-$, which may react with O_2 to produce $ONOO^-$, and ubisemiquinone (QH), part of which may react with O_2 to produce $·O_2^-$ [78]. Inhibition of complex I activity is not due to •NO itself but to species formed in tissue-specific mitochondrial pathways of •NO metabolism. $ONOO^-$ is likely to be the main species involved in damage of complex I, a view supported by the protective effects of superoxide dismutase and/or uric acid on complex I activity, the dose-dependent nitration of mitochondrial proteins observed upon exposure of mitochondrial membranes to •NO and the fact that the addition of $ONOO^-$ to submitochondrial particles resembles the effects of •NO [78].

Both •NO and O_2 are more soluble in lipid bilayers than in aqueous solutions and thus reach higher concentrations within cell membranes than soluble in cytosol. As the reaction between •NO and O_2 occurs much more rapidly within cell membranes, including mitochondrial membranes, than in the aqueous phases, part of the ability of mitochondria to breakdown added •NO may be due to this simple reaction within lipidic bilayers, which produces both $•NO_2$ and N_2O_3. Mitochondria can also increase •NO breakdown by reactions with superoxide, ubiquinol, and possibly cytochrome *c* oxidase, which might contribute to the regulation of •NO levels in cells [78]. Reversible •NO inhibition of respiration may result in local peroxynitrite production due to local superoxide production, causing irreversible inhibition of respiration and further oxidant production –a vicious cycle which might contribute to cell death.

In addition to stimulating H_2O_2 production, •NO or RNS can also inhibit catalase, deplete cellular glutathione and inhibit glutathione peroxidase, thus increasing H_2O_2 cellular levels. Indeed, •NO and H_2O_2 have been found to act synergistically in promoting cell death, possibly in part by superoxide dismutase-catalyzed peroxynitrite production [90]. •NO may also release iron from iron-sulfur centers and ferritin, potentially causing further oxidative stress.

RNS, S-nitrosothiols, or ROS cause MPT in isolated calcium-preloaded mitochondria [91]. MPT is a dramatic increase in permeability of the inner mitochondrial membrane to small (up to 1.5 kDa) molecules. Mitochondrial membrane potential and matrix calcium are known to determine the ability of other compounds to induce MPT; thus, inhibition of respiration by •NO and subsequent decrease in membrane potential should favor MPT opening. On the other hand, •NO itself can inhibit MPT due to direct inhibition of respiration, preventing calcium accumulation in mitochondria. cGMP formed by guanylyl cyclase after stimulation by •NO may also inhibit MPT via protein kinase G [85,92]. However, oxidants such as *tert*-butyl hydroperoxide and phenylarsine oxide at high concentrations can induce MPT even in the absence of calcium, an effect probably related to direct reaction of these compounds with functional thiols [93]. Therefore, •NO at high concentrations can promote MPT probably due to either (i) the production of peroxynitrite, nitrosothiols or $•NO_2/N_2O_3$, or (ii) depletion/oxidation of glutathione levels. •NO/RNS may directly oxidize the protein thiols which regulate the opening of the MPT pore.

MPT plays an important role in both necrotic and apoptotic cell death. MPT dissipates the protonmotive force, causing uncoupling of oxidative phosphorylation and reversal of the ATP synthase, potentially hydrolyzing cellular ATP resulting in necrosis. MPT also causes rapid swelling of the mitochondria, which may trigger outer membrane rupture and the release of intermembrane proteins like cytochrome *c*. However, MPT-related cytochrome *c* release in cells can occur by other mechanisms which do not involve mitochondrial swelling and membrane rupture. Release of cytochrome *c* and matrix components, such as NADH, inhibits respiration, potentially causing necrosis. In contrast, the release of cytochrome *c* and other apoptogenic intermembrane proteins, such as AIF and SMAC/Diablo, potentially triggers apoptosis [94]. Transient MPT opening may be a physiological process and usually does not cause cell damage, while longer sustained MPT opening may cause either apoptosis or necrosis [95]. The cellular death pathway triggered after MPT opening is likely to depend on additional factors, such as activation of Bid/Bax/Bad pathway or availability of ATP (ATP depletion probably favoring necrosis). Calcium has been suggested to cause cytochrome *c* release from mitochondria and subsequent apoptosis by stimulating mtNOS (mitochondrial NOS) to produce peroxynitrite, which then induces MPT or related processes.

10. •NO and peroxynitrite effects on mitochondrial superoxide and hydrogen peroxide production and on the PTP

Poderoso et al. [33] reported that the addition of •NO or •NO donors greatly increased superoxide production by submitochondrial particles, and also greatly increased hydrogen peroxide production by both isolated mitochondria and submitochondrial particles respiring on succinate. The half-maximal stimulation was at 0.3 μM •NO, a concentration which also half-inhibited complex III activity, and it was thus suggested that the inhibition of complex III caused superoxide and hydrogen peroxide production. The submitochondrial particles also caused rapid breakdown of added •NO, which was prevented by added superoxide dismutase, suggesting that the •NO-induced superoxide production might result in peroxynitrite production. Isolated heartperfusion with

•NO resulted in a rise in hydrogen peroxide in the perfusate, although this increase was not detected until 20 min after •NO perfusion. Radi et al. reported that peroxynitrite addition resulted in greatly increased hydrogen peroxide production by mitochondria (half-maximal stimulation at about 1.5 mM peroxynitrite) [96]. The •NO-induced superoxide and hydrogen peroxide production by mitochondria might result from (a) •NO interaction with complex III, as proposed by Poderoso and colleagues, (b) •NO inhibition of cytochrome *c* oxidase causing reduction of the respiratory chain, which is known to enhance superoxide production, or (c) •NO conversion to peroxynitrite, which enhances hydrogen peroxide production. If •NO enhances mitochondrial superoxide generation, and subsequent peroxynitrite production within the mitochondria, then this peroxynitrite might convert the initial reversible inhibition of respiration (due to •NO inhibition of cytochrome *c* oxidase) into irreversible inhibition (due to peroxynitrite inhibition of other complexes). Continuous exposure of mitochondria to 1 μM •NO for 15 min renders detectable peroxynitrite formation and results in an irreversible inhibition of respiration even after •NO has been removed. On the other hand, it is possible that the continuous production of peroxynitrite, at levels which are too low to detect, can still eventually cause significant inhibition. Peroxynitrite also inhibits the mitochondrial MnSOD by nitration, thus potentially preventing the breakdown of mitochondria-generated superoxide and favoring further generation of mitochondrial peroxynitrite. The •NO-induced hydrogen peroxide production might also be important in converting a reversible inhibition into an irreversible one, as •NO rapidly and reversibly inhibits catalase by binding to the heme group of catalase (Ki 0.2 μM •NO) [97]. •NO does not immediately inhibit glutathione peroxidase (the other main route for hydrogen peroxide breakdown), but it does exert inhibition over the time scale of an hour even at relatively low levels of •NO (supplied by a nitrosothiol), probably due to nitrosylation of a protein thiol. Thus, •NO may not only increase hydrogen peroxide production but also inhibit its breakdown.

Peroxynitrite rapidly oxidized mitochondrial glutathione to a variety of products, leading to pore opening. However, this permeability transition was prevented by either dithiothreitol or respiratory substrates that maintained the mitochondrial NAD(P)H in a reduced state [98]. Balakirev et al. found that •NO itself could both induce or inhibit PTP, the former effect resulting from secondary formation of superoxide or peroxynitrite, and the latter associated to •NO inhibition of cytochrome *c* oxidase and subsequent membrane depolarization [99]. Brookes et al. also observed that peroxynitrite increased mitochondrial proton permeability, possibly by peroxidation of mitochondrial phospholipids [100].

11. Exogenous or endogenous •NO-induced mitochondrial damage and cellular death

Abundant literature discusses •NO-related mitochondrial inhibition or damage in cells and tissues in relation to pathology. Much of this literature is difficult to interpret in terms of specific mechanisms because (a) agents other than •NO may be involved, (b) •NO may give rise to a variety of chemical products in cells, and (c) a variety of indirect effects may cause mitochondrial damage (e.g. calcium, free radicals, necrosis and apoptosis). However, it is clear that •NO and its products play an important role in inflammatory, ischemic and neurodegenerative pathologies, as well as in host defense against pathogens, with mitochondrial inhibition and damage being among means by which •NO exerts its cytostatic and cytotoxic effects.

Macrophages, activated by cytokines and endotoxins to express iNOS, can cause cytostasis and/or cytotoxicity in tumor and microbial cells, an important activity in non-specific host defense. Isolated macrophages or macrophage-derived cell lines have been co-cultured with tumor cell lines or a variety of microbial pathogens to investigate the mechanism of this toxicity, and as a model for inflammatory damage to host cells. Although activated macrophages produce a variety of potentially toxic substances, such as cytokines, superoxide and hydrogen peroxide, their cytostatic and toxic properties are mainly due to •NO production from iNOS. Indeed, inhibition of NOS or scavenging of •NO prevent cytotoxicity [101] and •NO-dependent activity is associated with a characteristic set of metabolic changes in both target tumor cells and macrophages, in particular inhibitions of mitochondrial aconitase, complex I, complex II and nuclear DNA synthesis, and the loss of intracellular iron [78,87]. These results are reproduced by incubating the cells anaerobically with •NO, which suggests that the changes are caused by the displacement of iron by •NO from the iron-sulfur centers of aconitase, complex I, complex II and ribonucleotide reductase (a rate-limiting enzyme in DNA synthesis). Tumor cells co-cultured with activated macrophages showed that some mitochondrial iron-sulfur proteins were inhibited before others, with aconitase being inhibited first, followed by complex I and then complex II, whereas complex III remained non-inhibited.

The findings that aconitase, complex I and complex II are inhibited by peroxynitrite but apparently not by •NO raise the question whether •NO-and iNOS-induced damage to mitochondria in cells might be mediated by peroxynitrite and not solely by •NO. However, this important point has not been fully elucidated, as high levels of •NO can partially inhibit complex II and aconitase.

Szabo et al. found that incubating macrophage cell line J774 with •NO donors, a peroxynitrite generator (SIN-1), peroxynitrite or endotoxin/interferon-γ to induce iNOS caused partial inhibition of this succinate dehydrogenase activity in these cells. A peroxynitrite scavenger partially protected the enzymatic activity from inactivation by peroxynitrite but not by •NO donors, and also moderately protected the activity of succinate dehydrogenase from inactivation by endotoxin/interferon [102].

Irreversible inhibition of respiration in macrophages treated with •NO donors DETA-NONOate occurred after a delay of several hours, at a time when cellular glutathione levels were depleted, and appeared to be mainly due to the inhibition of complex I. The inhibition could not be prevented by peroxynitrite or superoxide scavengers, whereas, surprisingly, both the inhibition of respiration and complex I could be completely reversed by either light or thiol-reducing reagents such as DTT. This suggests that the inhibition was due to direct nitrosylation of thiols in complex I, which are reversed by light or reducing agents [40].

In a variety of other cellular types, induction of iNOS causes irreversible inhibition of mitochondrial respiration, usually associated with an inhibition of aconitase, complex I and/or complex II. For instance, in hepatocytes, iNOS induction has been associated with inhibition of mitochondrial aconitase and respiration based on citrate, malate and succinate as metabolic fuels, and a fall in cellular ATP content. Pretreatment of hepatocytes with high levels of •NO (about 200 μM) in anaerobic conditions for 5 min resulted in inhibition of cellular respiration, mitochondrial aconitase, complex I and complex II, but no inhibition of cytosolic aconitase, complex III or complex IV. The •NO-induced inhibition of mitochondrial aconitase and complexes was largely reversible by incubating the cells in the absence of •NO [86,103]. In turn, exposure of hepatocytes to •NO donors in the presence of O_2 causes immediate depolarization of the mitochondrial membrane, an increase in cytosolic calcium levels and rapid cell death, which is prevented by cyclosporin A, probably caused by inhibition of cytochrome *c* oxidase and by the opening of the mitochondrial PTP. Hepatocytes incubated or co-cultured with activated macrophages or Kupffer cells (resident liver macrophages) show inhibition of aconitase and mitochondrial respiration on all substrates. However, induction of iNOS in the Kupffer cells *in vivo*, which causes a dramatic increase in •NO production, did not lead to any irreversible inhibition of aconitase, mitochondrial respiration or the appearance of mitochondrial iron-nitrosyl complexes, although it did cause liver necrosis [104]. This important observation suggests that, *in vivo*, the scavenging of •NO by oxyhemoglobin in blood is sufficient to prevent or reverse •NO inactivation of mitochondrial iron-sulfur centers.

In fibroblasts, endotoxin/interferon-γ activation of iNOS expression caused inhibition of malate- and succinate-dependent respiration by an L-arginine-dependent process. In cardiac myocytes, cytokine-induced expression of iNOS caused inhibition of succinate dehydrogenase, as measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and this inhibition was reversed by inhibiting iNOS with NMA or by removing the cytokines.

In brain, endotoxin/interferon-induced iNOS expression in cultured astrocytes or microglia caused irreversible inhibition of complex IV, a less pronounced inhibition of complex II–III activity, •NO inhibition of complex I or citrate synthase and a stimulation of glycolysis, all these effects being preventable by NOS inhibition. The irreversible inhibition of cytochrome *c* oxidase was partially prevented by adding high concentrations of Trolox, a vitamin E analog and anti-oxidant, which suggests the involvement of lipid peroxidation, perhaps peroxynitrite. The addition of peroxynitrite to cultured neurons caused inhibition of complexes II–III and complex IV, but not complex I, measured 24 h after peroxynitrite addition, accompanied by cell death. However, addition of up to 2 mM peroxynitrite to astrocytes caused no inhibition of mitochondrial complexes. Induction of iNOS in astrocytes also causes a potent and reversible inhibition of cell O_2 consumption due to acute •NO inhibition of cytochrome *c* oxidase in competition with O_2. This inhibition can be immediately reversed by inhibiting iNOS or binding •NO to hemoglobin.

Brief exposure to low levels of •NO is cytotoxic to neurons by mechanisms which are not entirely clear but may involve inhibition of mitochondrial respiration. •NO potently and reversibly inhibits the mitochondrial respiration of isolated nerve terminals and causes release of neurotoxic neurotransmitter glutamate from these terminals due to •NO inhibition of cytochrome *c* oxidase. The neurotoxicity of •NO may partially, depending on conditions, be due to the •NO-induced glutamate release, which is then toxic to neurons via NMDA receptors [105]. This toxicity may be enhanced when •NO is inhibiting mitochondrial respiration and oxidative phosphorylation, as (a) this will cause depolarization of the plasma membrane and thus dramatically increase the sensitivity of the NMDA receptor to glutamate, and (b) this will depolarize the mitochondrial membrane and thus reduce the buffering of cytosolic calcium. Expression of iNOS in mixed astrocytic-neuronal cocultures causes delayed neuronal death and inhibition of mitochondrial complexes II–III and complex IV, but not complex I or citrate synthase in neurons. The same pattern of mitochondrial inhibition is caused by adding peroxynitrite to neurons, which indicates that iNOS expression in astrocytes/microglia may inhibit the respiration of co-cultured neurons via peroxynitrite.

•NO can induce apoptosis in some cell types, including macrophages, thymocytes, T cells, myeloid cells and neurons, and this may be mediated by the effects of •NO on mitochondrial respiration. Addition of •NO to these cells causes inhibition of respiration, depolarization of the mitochondrial membrane potential, release of mitochondrial apoptogenic factors into the cytosol, activation of caspases and thus apoptosis. However, things are not always this simple. •NO can promote or block apoptosis –partly by inhibiting the caspases– and either promote or inhibit necrosis, depending on conditions [106–108]. The decision between apoptosis and necrosis may partly depend on ATP levels, as a marked drop in ATP can block apoptosis but promote necrosis. The level of cellular thiols such as glutathione is also important, since thiols scavenge peroxynitrite and can reverse many of •NO and peroxynitrite effects, partly by re-reducing oxidized and nitrosylated protein thiols. The presence of glucose and rate of glycolysis can also play a part, as cells can survive the inhibition of mitochondrial respiration caused by induction of iNOS as long as glucose is present and glycolysis is active to maintain ATP levels. The level of O_2 is also a key player in determining whether and how cells die in response to •NO, as it affects the sensitivity of cytochrome *c* oxidase to •NO and the rate of breakdown of •NO to $•NO_2$ and N_2O_3.

Table 1
Mitochondrial components affected by •NO, peroxynitrite or iNOS expression.

Component affected	Effector	Nature of effect
Cytochrome *c* oxidase	•NO	50–500 nM causes reversible inhibition competitive with O_2
Cytochrome *c* oxidase	Peroxynitrite	Irreversible inhibition (> 2 mM)
Cytochrome *c* oxidase	iNOS expression	Inhibition in astrocytes
Cytochrome c^{3+}	•NO	Reversible binding, Kd 20 μM
Cytochrome c^{2+}	•NO	Reaction producing cyt c^{3+} and $•NO^{-2}$
Cytochrome c^{2+}	Peroxynitrite	Reaction producing cyt c^{3+}
Complex III	•NO	Reversible inhibition
Ubiquinol	•NO	Reaction
Complex II	•NO	2 mM •NO removes iron-sulfur center
Complex II	Peroxynitrite	Irreversible inhibition
Complex II	iNOS expression	Macrophages and target tumor cells
Complex II	•NO in cells	Inhibition after incubation with high levels
Complex I	iNOS expression	Macrophages and target tumor cells
Complex I	•NO in cells	Inhibition after incubation with high levels
Complex I	Peroxynitrite	Inhibition
NADH	Peroxynitrite	Oxidation and hydrolysis
Aconitase	•NO	Partial inhibition by high levels
Aconitase	Peroxynitrite	Inhibition reversible by DTT plus iron
Aconitase	iNOS	Inhibition
Aconitase	•NO in cells	Inhibition
Creatine kinase	•NO	Inhibition reversible by DTT
Creatine kinase	Peroxynitrite	Irreversible inhibition
ATP synthase	Peroxynitrite	Inhibition
Proton and ion leaks	Peroxynitrite	Increase

12. Discussion

The effects of •NO and peroxynitrite on mitochondria are clearly distinct and should be distinguished (see Table 1). •NO causes reversible and relatively specific inhibition of cytochrome *c* oxidase. However, high levels of •NO for long periods can cause other effects which may be mediated by the reversible nitrosylation of protein thiols and probably by the removal of iron from iron-sulfur centers. Peroxynitrite, by contrast, potentially oxidizes most of the components in the mitochondria, causing the oxidation and cross-linking of proteins, inhibition of most of the mitochondrial complexes, nitration of tyrosine residues, oxidation of non-protein thiols, oxidation of membrane lipids and disruption of the mitochondrial membrane. Unfortunately, the effects of $•NO_2$ and N_2O_3 on mitochondria are still unclear. While their production rates were speculated to be low, the finding that the reaction between •NO and O_2 is much faster within bilayer membranes suggests that $•NO_2$ and N_2O_3 may be more important in •NO-induced toxicity than previously considered. The effects of the nitroxyl ion and nitrosothiols on mitochondria are also relatively unclear.

The mechanisms by which •NO may kill or protect cells are also still to be elucidated, as a variety of mechanisms appear to be at work depending on different cellular conditions. Nevertheless, one of the main mechanisms involved is the generation of peroxynitrite from •NO and the subsequent damage to mitochondria ultimately leading to cellular necrosis or apoptosis. Peroxynitrite also damages non-mitochondrial targets, including nuclear DNA, setting up the conditions for cellular death. Evidence that peroxynitrite mediates mitochondrial damage in a variety of pathologies and •NO cytotoxicityis still inconclusive. •NO can drive cells to death by mechanisms independent of peroxynitrite but, in many models of •NO-induced cytotoxicity, peroxynitrite seems most likely to be the *villain* of the story.

Abbreviations

cGMP cyclic guanosine monophosphate
eNOS endothelial nitric oxide synthase

DTT	dithiothreitol
DETA-NONOate	(2,2′-(hydroxynitrosohydrazino) bis-ethanamine
iNOS	inducible nitric oxide synthase
MPT	mitochondrial permeability transition
NMDA	N-methyl-D-aspartate
nNOS	neuronal nitric oxide synthase
PTP	permeability transition pore
RNS	reactive nitrogen species
ROS	reactive oxygen species
TMPD	N,N,N′,N'-tetramethyl-p-phenylenediamine

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.niox.2019.04.005.

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