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Review

Nitric oxide and mitochondrial respiration

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Abstract

Nitric oxide (NO) and its derivative peroxynitrite ($ONOO^-$) inhibit mitochondrial respiration by distinct mechanisms. Low (nanomolar) concentrations of NO specifically inhibit cytochrome oxidase in competition with oxygen, and this inhibition is fully reversible when NO is removed. Higher concentrations of NO can inhibit the other respiratory chain complexes, probably by nitrosylating or oxidising protein thiols and removing iron from the iron-sulphur centres. Peroxynitrite causes irreversible inhibition of mitochondrial respiration and damage to a variety of mitochondrial components via oxidising reactions. Thus peroxynitrite inhibits or damages mitochondrial complexes I, II, IV and V, aconitase, creatine kinase, the mitochondrial membrane, mitochondrial DNA, superoxide dismutase, and induces mitochondrial swelling, depolarisation, calcium release and permeability transition. The NO inhibition of cytochrome oxidase may be involved in the physiological regulation of respiration rate, as indicated by the finding that isolated cells producing NO can regulate cellular respiration by this means, and the finding that inhibition of NO synthase in vivo causes a stimulation of tissue and whole body oxygen consumption. The recent finding that mitochondria may contain a NO synthase and can produce significant amounts of NO to regulate their own respiration also suggests this regulation may be important for physiological regulation of energy metabolism. However, definitive evidence that NO regulation of mitochondrial respiration occurs in vivo is still missing, and interpretation is complicated by the fact that NO appears to affect tissue respiration by cGMP-dependent mechanisms. The NO inhibition of cytochrome oxidase may also be involved in the cytotoxicity of NO, and may cause increased oxygen radical production by mitochondria, which may in turn lead to the generation of peroxynitrite. Mitochondrial damage by peroxynitrite may mediate the cytotoxicity of NO, and may be involved in a variety of pathologies.

Keywords: Mitochondria; Respiration; Nitric oxide; Peroxynitrite; Cell death; Oxygen

Contents

Abbreviations: cyt, cytochrome; DETA-NONOate, 2,2′-(hydroxynitrosohydrazino)bisethanamine; DTT, dithiothreitol; EPR, electron paramagnetic resonance; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; iNOS, inducible isoform of nitric oxide synthase; MnTBAP, Mn(III)tetrakis(4-benzoic acid) porphyrin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMA, N^{ω}-nitro-L-arginine; NMDA, *N*-methyl-D-aspartate; NMMA, N^{ω}-monomethyl-L-arginine; NOS, nitric oxide synthase; PARS, poly(ADP) ribosyltransferase; PTP, permeability transition pore; SIN-1, 3-morpholinosydnonimine-*N*-ethylcarbamide; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine

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1. Introduction

This article reviews the interactions between nitric oxide (NO) and mitochondrial respiration. The question is: why would you want to know anything about NO and mitochondrial respiration? Mitochondrial respiration is responsible for virtually all energy production in mammals, and every other process in living organisms ultimately depends on energy production [1]. Furthermore, both necrosis and apoptosis, that is most forms of cell death, are intimately linked to mitochondrial function [2,3].

Joseph Priestley discovered both nitric oxide ('nitrous air') and oxygen ('dephlogistionated air') in the early 1770s, and used the reaction between these two gases, producing the soluble, brown gas nitrogen dioxide, to quantify the amount of oxygen in normal air [4]. And he applied this method to show that mice consumed the oxygen of the air, and thus developed the first accurate measurement of respiration. NO was subsequently used as a chemical tool, but it was not until 1987/1988 that it was recognised that NO was produced and used by animals as both a physiological regulator and cytotoxic agent [5–7].

The paradox of NO acting as both a physiological regulator and a cytotoxic agent was apparently resolved when it was found that NO reacts with superoxide to produce peroxynitrite ($ONOO^-$), which is a potent oxidant and cytotoxic agent. Thus, it was suggested that NO was mostly responsible for the physiological regulation, while peroxynitrite was responsible for the cytotoxic properties [8]. However, this appealing dichotomy may not be so clear cut as once hoped.

Even before NO was discovered to be the responsible agent, it was known that activated macrophages produced an agent that was cytotoxic to other cells by irreversibly inhibiting their mitochondrial respiration [6,7,9–14]. NO, and its derivatives peroxynitrite and nitrogen dioxide, can indeed irreversibly inhibit mitochondrial respiration, but in 1994 a radically different effect of NO on mitochondrial respiration was reported [15–17]. Very low (nanomolar) levels of NO caused a completely reversible inhibition of mitochondrial respiration at cytochrome oxidase in competition with oxygen. This raised the exciting possibility that NO was a physiological regulator of mitochondrial respiration [18,19], in fact the only known direct regulator of respiration [20]. Below I will review these two very different, reversible and irreversible, effects of NO on mitochondrial respiration. A variety of other reviews related to this subject have been published recently [21–24].

2. Reversible NO inhibition of cytochrome oxidase

Cytochrome *c* oxidase (cytochrome aa_3, complex IV) is the terminal complex of the mitochondrial respiratory chain, responsible for about 90% of oxygen consumption in mammals, and essential for virtually all energy production in cells [25]. It is located in the mitochondrial inner membrane, and catalyses the oxidation of cytochrome c^{2+} to cytochrome c^{3+} and the reduction of oxygen to water, and this is coupled to the pumping of protons out of the mitochondria (see Fig. 1). The oxidase contains two haems (cyt *a* and cyt a_3) and two copper centres (CuA and CuB). Oxygen binds to the reduced form of a binuclear centre consisting of cytochrome a_3 (Fe^{2+}) and CuB (Cu^{+}) within the complex, and this constitutes the oxygen binding site and catalytic centre of the oxidase [25].

NO binds to the oxygen binding site of cytochrome oxidase, and this was first reported in 1955 by Wainio [26], who found that NO added to the reduced form of isolated cytochrome oxidase induced a shift in the optical spectrum of cytochromes aa_3 similar to the shift induced by carbon monoxide. This finding was subsequently confirmed and extended [27–32], and indicates that NO binds to the same site and form of the enzyme as oxygen, i.e. the reduced form of haem a_3. This raises the possibility that NO is an inhibitor of cytochrome oxidase in competition with oxygen. That NO is indeed an inhibitor of cytochrome oxidase turnover was first reported in 1994 [15–17], although Carr and Ferguson [33] had earlier shown that the product of nitrite and nitrite reductase (presumed to be NO) inhibited the oxygen consumption of bovine heart submitochondrial particles (inner mitochondrial membranes). Brown and Cooper [15] showed that 1 μM NO added to isolated cytochrome oxidase caused an immediate inhibition of oxygen consumption that was completely reversed when the NO was broken down. Thus NO is a potent, rapid and reversible inhibitor of cytochrome oxidase. Brown and Cooper [15] also showed that NO reversibly inhibited the oxygen consumption of isolated synaptosomes, which are neuronal nerve terminals isolated from the brain, and are packed with neuronal mitochondria. They found [15] that the NO inhibition of oxygen consumption was competitive with oxygen, half-inhibition of synaptosomal oxygen consumption occurred at 270 nM NO when the oxygen concentration was about 145 μM O_2 (roughly the arterial level of oxygen), but half-inhibition occurred at 60 nM NO when the oxygen concentration was 30 μM (roughly the mean tissue level of oxygen). Borutaite and Brown [34] reported that similar levels of NO reversibly inhibited respiration in isolated heart mitochondria due to the inhibition of cytochrome oxidase, but oxygen consumption was markedly less sensitive to NO in state 4 than in state 3 [34], for reasons that are not entirely clear. Cassina and Radi [35] reported that somewhat higher levels of NO were required to inhibit oxygen consumption in heart mitochondria, but they did not

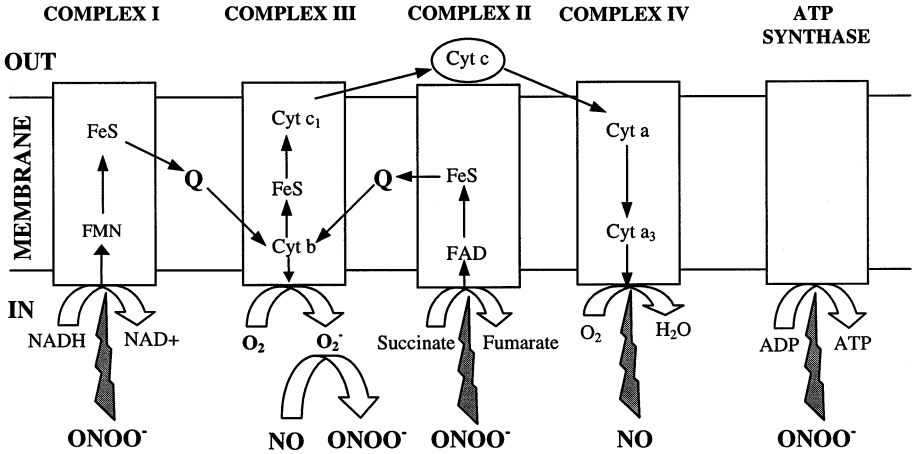

Fig. 1. Schematic diagram of the principle actions of NO and peroxynitrite on the mitochondrial complexes. The five mitochondrial complexes are located within the mitochondrial inner membrane. Electron pathways are represented by light arrows, and inhibitions are depicted as thunderbolts.

actually measure the levels of NO. Poderoso et al. [36] found that 0.1 μM NO half-inhibited cytochrome oxidase activity in submitochondrial particles, and 0.5 μM NO half-inhibited oxygen consumption in heart mitochondria at unspecified oxygen levels (probably high) [36,37]. Takehara et al. [38] and Nishikawa et al. [39] reported that addition of about 1 μM NO to isolated liver mitochondria caused reversible inhibition of oxygen consumption and ATP synthesis which was greater at lower oxygen levels. Cleeter et al. [16] showed that a NO donor caused a reversible inhibition of oxygen consumption in isolated skeletal muscle mitochondria due to inhibition of cytochrome oxidase. Lizasoain et al. [40] demonstrated that NO donors reversibly inhibited the respiration of submitochondrial particles at cytochrome oxidase with an apparent half-inhibition by 2 μM NO. Schweizer and Richter [17] found that about 1 μM NO caused a reversible inhibition of oxygen consumption in isolated liver and brain mitochondria, and this resulted in a reversible depolarisation of the mitochondrial membrane potential and efflux of mitochondrial calcium, which was greater at lower oxygen concentrations.

In isolated cytochrome oxidase (from beef heart) Torres et al. [41] and Giuffre et al. [42] found NO binding to the oxygen binding site was competitive with oxygen, and due to NO binding to a partially reduced form of the cyt a_3-CuB binuclear centre. Thus, in the presence of oxygen during turnover of cytochrome oxidase, addition of NO resulted in an optical spectrum identical to that of the oxidase-NO complex in the absence of oxygen. The levels of NO required for inhibition were somewhat higher than those reported by Brown and Cooper [15], but again the actual levels of NO present were not measured [41,42]. NO is known to bind rapidly to the Fe^{2+} of cyt a_3 ($k = 0.4–1.0 \times 10^8$ M^{-1} s^{-1}) [32,43], and NO also dissociates surprisingly rapidly ($k = 0.13$ s^{-1}) [42]. But oxygen also binds rapidly to the Fe^{2+} of cyt a_3 ($k = 10^8$ M^{-1} s^{-1}) [25], and at high ratios of oxygen to NO it appears anomalous that NO can inhibit cytochrome oxidase so rapidly (less than 1 s) by binding to cyt a_3 alone [41,42]. Thus Torres et al. [41] proposed that during turnover, NO preferentially binds to the reduced form of CuB (Cu^+), which then somehow gives NO a kinetic advantage over oxygen in binding to the Fe^{2+} of cyt a_3. They subsequently suggested and provided evidence that NO binds to CuB^{2+}, forming Cu^+-NO^+, 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 the electron may be passed to cyt a_3, cyt a or CuA [44,45]. This binding of NO to Cu^+ may be the major pathway for rapid inhibition of the oxidase [45]. However, Giuffre et al. [42] believe that the rapid onset of inhibition may still be explained in terms of rapid binding of NO to the Fe^{2+} of the partially reduced binuclear centre. Brudvig et al. [30] had previously studied the reactions of NO with isolated cytochrome oxidase in some detail. And Cassina and Radi [35] had shown that NO can reduce the fully oxidised oxidase in mitochondria (in the presence of antimycin) generating a nitrosyl-(Fe^{2+})-haem a_3.

The affinity of NO for cytochrome oxidase is somewhat lower (about 5-fold lower) when measured by the NO-induced absorbance change of isolated cytochrome oxidase [41,42], as compared to when measured by the inhibition of mitochondrial respiration [15,34,36,38,46]. This discrepancy might have a number of causes, including the fact that light (which is used to measure the absorbance change) can dissociate NO from the oxidase [31]. Indeed we have recently found that light can reverse the NO inhibition of mitochondrial respiration (V. Borutaite, G.C. Brown, unpublished data).

NO also potently inhibits the cytochrome oxidase activity of the bacteria *Paracoccus denitrificans* [33] and *Escherichia coli* [47], as well as that of higher plant mitochondria [48]. Some bacterial cytochrome oxidases are homologous to the nitric oxide reductase of denitrifying bacteria, leading to the suggestion that cytochrome oxidase may have evolved from denitrifying enzymes [49]. Mitochondrial cytochrome oxidase may in fact have some residual nitric oxide reductase activity [30,34,50,51] as well as nitric oxide oxidase activity [30,45], which may contribute to the ability of mitochondria and cells to breakdown NO [34,52]. However, Stubauer et al. [53] recently reanalysed the ability of isolated cytochrome oxidase to reduce NO, but found no such activity. Also it was recently reported that phospholipid bilayers can catalyse the oxidation of NO to NO_2, due to the fact that both NO and O_2 are more soluble in the bilayer

than the water phase [54], and thus mitochondria may catalyse NO oxidation partly due to their phospholipid membranes catalysing this reaction.

Because NO competes with oxygen at cytochrome oxidase, NO acts to increase the apparent K_m of respiration for oxygen [15], and this led Brown [18] to suggest that NO may be a physiological regulator of the oxygen sensitivity of respiration in tissues. Koivisto et al. [46] reinvestigated the competition between NO and oxygen in isolated mitochondria from brown adipose tissue, and found that half-inhibition of respiration occurred at 364 nM NO with 180 μM O_2, 69 nM NO with 72 μM O_2, and 11 nM NO with 32 μM O_2. Thus the IC_{50} of NO increased roughly in proportion to the square of the oxygen tension, and in the presence of NO the dependence of respiration on oxygen tension had a Hill coefficient of about 2. They pointed out that this was consistent with two molecules of NO competing with one molecule of O_2 [46]. Since the average level of oxygen in mammalian tissues is about 30 μM O_2, these results emphasise that even low physiological levels of NO (1–200 nM NO) can cause substantial inhibition of respiration, and potentially make tissue respiration very sensitive to the oxygen tension [18].

The cell membrane is permeable to NO, and thus NO (or NO donors) added to cells causes rapid and oxygen-dependent inhibition of respiration at cytochrome oxidase in neuronal nerve terminals [15,55,56], hepatocytes [57], astrocytes [58,59], pancreatic β-cells [60], and ascites tumour cells [61]. This respiratory inhibition was largely reversed when the NO was removed, although there also was an irreversible component to the inhibition in some cases [58,59]. The reversible inhibition of respiration by NO resulted in a reversible and oxygen-dependent decrease in mitochondrial membrane potential and increase in cytosolic calcium (probably due to efflux of mitochondrial calcium) in hepatocytes [57], β-cells [60], and tumour cells [61], and additionally a decrease in cellular ATP in ascites tumour cells [61]. NO-induced respiratory inhibition was also accompanied by insulin release by β-cells and intact pancreatic islets [60] and glutamate release by nerve terminals [56]. NO donors also inhibited the cellular oxygen consumption and decreased the ATP level of lung alveolar type II cells [62].

However, the above findings that addition of NO to in vitro system causes inhibition of respiration at cytochrome oxidase is not sufficient to show that NO is a physiological regulator or pathological inhibitor of mitochondrial respiration. For this we need to know whether NO produced endogenously by NO synthase in cells causes reversible inhibition of cellular respiration. Such research is reviewed in Section 3.

3. Reversible inhibition of mitochondrial respiration by NO endogenously produced by cells, tissues, and in vivo

Investigation of whether NO can regulate respiration in cells, tissues and in vivo is important but complicated by several factors. (1) NO can react with cellular constituents to produce peroxynitrite, nitrosothiols and other derivatives of NO, which may affect respiration but generally cause an irreversible inhibition. (2) NO may affect ATP consumption (or respiratory substrate supply), which will indirectly change cellular oxygen consumption, in particular NO can affect muscle contraction by cGMP-dependent mechanisms [63,64]. (3) NO is a potent vasodilator and thus increases oxygen supply. (4) NO reacts rapidly with haemoglobin and myoglobin. Thus these experiments can be difficult to interpret mechanistically.

A variety of cells, including macrophages, astrocytes, hepatocytes, and myocytes, can be induced to express the inducible form of NO synthase (iNOS) by cytokines, endotoxins, and/or oxidative stress, leading to the production of a sustained high level of NO [7,58,65–67]. Primary cultures of astrocytes isolated from brain were activated to express iNOS by interferon-γ and endotoxin and found to produce up to 1 μM NO, and this endogenous NO was found to cause potent inhibition of cellular respiration at cytochrome oxidase, which was rapidly reversed in part by either removing the NO with oxyhaemoglobin or by inhibiting NO synthase [58]. Recently we (G.C. Brown, N. Foxwell, S. Moncada, unpublished data) have also shown that a macrophage cell line (J774) activated to express iNOS can cause inhibition of the cellular respiration of co-incubated cells via the reversible NO inhibition of cytochrome oxidase. Furthermore we [155] have recently found that primary

cultures of endothelial cells, expressing the constitutive, endothelial form of NO synthase (eNOS), when stimulated with bradykinin or ATP, release a brief pulse of NO, which causes a brief, reversible inhibition of cellular respiration due to the inhibition of cytochrome oxidase. Also the background, unstimulated release of NO was sufficient to partially inhibit endothelial cell respiration, so that inhibition of NOS caused a stimulation of respiration. Miles et al. [62] found that inhibition of NO synthase in lung alveolar type II cells caused an increase in cellular oxygen consumption and ATP concentrations, while an NO donor caused the opposite effect, suggesting that constitutive NO production was inhibiting respiration. Shen et al. [19] found that addition of the endothelial agonists bradykinin or carbachol to in vitro slices of skeletal muscle caused NO-dependent inhibition of oxygen consumption. A cell-permeable form of cGMP (8-bromo-cGMP) also caused an inhibition of oxygen consumption by the muscle slices, suggesting that part of the inhibition may have been mediated by cGMP. However, in the presence of a mitochondrial uncoupler (dinitrophenol) bromo-cGMP did not inhibit respiration, while bradykinin still inhibited respiration, suggesting that at least part of the inhibition was due to a direct effect of NO on the respiratory chain [19]. In heart muscle slices incubated in vitro the same authors found [68] that bradykinin and carbachol caused an NO-dependent inhibition of tissue respiration that was not mimicked by cell-permeable cGMP and also occurred in the presence of a mitochondrial uncoupler. This suggests that endothelial-derived NO directly inhibited the mitochondrial respiratory chain or substrate supply. However, the reversibility and oxygen dependence of this inhibition were not measured, so we cannot be sure that it was due to NO inhibition of cytochrome oxidase. Poderoso et al. [37] used isolated, perfused rat heart and showed that bradykinin caused a 30–40% decrease in oxygen consumption, associated with an increase in NO concentration in the effluent, vasodilation of the coronary vasculature, but no significant decrease in left ventricular pressure. These effects were blocked by inhibiting NO synthase with *N*-monomethyl-L-arginine. These results suggest that bradykinin-evoked NO release from the endothelium can inhibit myocyte oxygen consumption, but again the reversibility, oxygen and cGMP dependence of the inhibition were not measured, so the mechanism of the inhibition is unclear.

In vivo in conscious dogs Shen et al. [69] found that an inhibitor of NO synthase (NMA, *N*-nitro-L-arginine) caused a rapid and sustained 25% increase in whole body oxygen consumption (estimated from cardiac output and oxygen extraction), even though the oxygen available to tissues was decreased due to the haemodynamic changes. Barbiturate (pentobarbital) anaesthetised dogs did not show the NMA-induced stimulation of oxygen consumption. The same authors [19] found that NMA also increased the oxygen consumption in hind limb skeletal muscle of conscious dogs by 55%. And the same authors using the same model found that NMA increased the oxygen consumption of the kidneys by 58% in vivo, though sodium reabsorption was decreased, suggesting that kidney respiration was inhibited directly by endogenous NO [70]. They also found that bradykinin or NO donors decreased the oxygen consumption renal slices in vitro [70]. King et al. [71] also found that NOS inhibition caused a 40% increase in the oxygen consumption of hind limb skeletal muscle of anaesthetised dogs, despite a decrease in blood flow. Ishibashi et al. [72] found that NMA caused a 28% increase in heart oxygen consumption of exercising dogs.

All of these results are consistent with the interpretation [19,68–71] that basal constitutive NO release by capillary endothelium or by NOS within muscle cells tonically inhibits tissue respiration. However, it cannot be ruled out that this inhibition is mediated via cGMP and/or ATP consumption, as cGMP can weakly inhibit muscle oxygen consumption in vitro [19], and possibly in vivo in rabbit hearts [73,74], and can inhibit isolated myocyte contraction [63,64] and oxygen consumption [75].

In contrast to the concept that cGMP can inhibit muscle and heart oxygen consumption, it has recently been reported that cGMP and NO donors can stimulate the oxidation of glucose, pyruvate, palmitate and leucine in isolated soleus muscle via cGMP-dependent mechanism [76–78]. 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 phos-

phorylating uncoupling proteins in the mitochondria [78]. However, the mechanism of the in vitro cGMP-dependent stimulation remains unclear – possibilities include increased perfusion of the muscle, or stimulation of ATP utilising processes in the muscle. Clearly more work needs to be done to untangle the multiple effects of NO/cGMP on respiration within tissues and in vivo.

4. Effects of NO on mitochondria not mediated by cytochrome oxidase

Poderoso et al. [13] reported that NO inhibited the cytochrome bc_1 complex (complex III) in submitochondrial particles. The inhibition was partially reversible, and occurred at NO concentrations somewhat higher than those that inhibit cytochrome oxidase. However, it is not clear that the inhibition was not 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. [79] reported in an abstract that NO can react directly with ubiquinol-2, an analogue of the endogenous mitochondrial electron transport carrier ubiquinol-10, and this might in part mediate the NO inhibition complex III activity or NO induction of mitochondrial superoxide production.

Welter et al. [80] treated purified cytochrome bc_1 complex (complex III), or purified succinate-ubiquinine reductase (complex II), or purified complexes II and III together as a supercomplex, with 2 mM NO for 1 min in strictly anaerobic conditions. This very high level of NO in the absence of oxygen resulted in a small (40%) inhibition of the activity of complex III when this complex was present alone, but no inhibition of complex III when present together with complex II in a supercomplex. The inhibition was accompanied by partial loss of the EPR signal of the Rieske iron-sulphur complex of complex III, but both this signal and complex III activity slowly recovered (over 12 h) to within 95% of the untreated control levels when the NO was removed. Welter et al. [80] suggested that high levels of NO induce a reversible change of structure of the Rieske iron-sulphur centre, without loss of the iron, but that complex III is protected from NO when in a supercomplex with complex II. By contrast Welter et al. [80] found that the above treatment with very high levels of NO in the absence of oxygen resulted in the total inhibition of complex II activity and the total loss of EPR visible iron-sulphur centres from complex II with the release of the iron as EPR-visible iron-NO. Thus NO itself has little effect on complex III, but very high concentrations of NO can irreversibly inhibit complex II by destroying iron-sulphur centres.

Cassina and Radi [35] found that treatment of rat heart mitochondria with 5 μM NO in the presence of oxygen resulted in an inhibition of respiration when respiring on succinate that was only partially reversible by removing the NO, whereas respiration on NADH-linked substrates (glutamate and malate) was almost completely recovered when the NO was removed. This irreversible inhibition of respiration on succinate by high levels of NO might be due to NO-induced destruction of complex II (as above) or due to peroxynitrite (or other oxides of NO) formation that may then inhibit complex II (see below).

Keilin [81], the discoverer of cytochromes, found that NO binds to cytochrome *c*. NO reversibly binds to oxidised (ferri-) cytochrome *c*, but the rate of binding is low (10^3 M^{-1} s^{-1}) and the dissociation constant is high (20 μM), so this would only be significant at very high levels of NO [82]. 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 oxygen to produce peroxynitrite [82]. This might possibly be a significant source of peroxynitrite independent of superoxide production [82], and the nitroxyl ion itself can be cytotoxic [83].

Creatine kinase, which buffers cellular ATP levels, is slowly inhibited by NO, probably via *S*-nitrosylation, and the inhibition is reversed by dithiothreitol [84,85]. In contrast, peroxynitrite rapidly and potently inhibits creatine kinase via oxidation of protein thiols, and this inhibition is not reversed by glutathione [86].

5. Mitochondrial NO synthase

The three known isoforms of NO synthase are found either in the cytosol or bound to a variety of membranes, depending on conditions and cell type.

Monoclonal antibodies to eNOS have been found to bind to mitochondria in cells and isolated mitochondria from a variety of tissues, including skeletal muscle, heart, liver, and kidney [87–89]. However, it is not clear how specific these antibodies are [90]. Kobzik et al. [87] found eNOS antibodies added to skeletal muscle slices co-localised with mitochondria, and mitochondria isolated from diaphragm muscle exhibited a calcium-dependent conversion of L-arginine to L-citrulline, and an L-arginine-dependent inhibition of oxygen consumption, consistent with NOS being active in mitochondria. Ghafourifar and Richter [90] found a calcium- and L-arginine-dependent NOS activity in isolated rat liver mitochondria and submitochondrial particles, and a NOS-dependent inhibition of oxygen consumption. They suggested that mitochondria contain a new isoform of NOS located on the inner mitochondrial membrane, and functioning to regulate mitochondrial respiration and calcium, and the activity of this NOS was possibly in turn regulated by mitochondrial matrix calcium [90].

Giulivi et al. [91] measured NO production by percoll-purified liver mitochondria using either oxymyoglobin or an EPR spin trap to measure NO. They found an arginine-dependent and NMMA-inhibitable NO production of 1.4 nmol/min/mg protein in intact mitochondria, and this activity was greater in sub-mitochondrial particles, suggesting that the NOS was located on the inner mitochondrial membrane. Tatoyan and Giulivi [92] purified the protein responsible for this activity to homogeneity from the same liver mitochondria, and showed that it was similar or identical to iNOS based on kinetic parameters, molecular weight, requirement for cofactors, and cross-reactivity to monoclonal antibodies to iNOS from macrophages. Whether this mitochondrial NOS (mtNOS) is in fact a distinct isoform of NOS is yet to be determined. Giulivi [93] further investigated the functional implications of this NO production for mitochondrial respiration in isolated liver mitochondria. It was found that the endogenously produced NO was dependent on the presence of L-arginine, was inhibited by *N*-methyl-L-arginine, and was dependent on the respiratory state of the mitochondria in such a way as to suggest that the NO production was supported by mitochondrial NADPH. The endogenous NO significantly inhibited state 4 and state 3 respiration and ATP production [93]. Thus, the high activity of mtNOS, location on the inner mitochondrial membrane, and demonstration that it can regulate mitochondrial respiration suggest that it may in fact function to regulate mitochondrial respiration physiologically.

6. Peroxynitrite-mediated mitochondrial inhibition

NO reacts rapidly with superoxide (O_2^-) to produce peroxynitrite ($ONOO^-$), which may act as an oxidant itself, or isomerise to nitrate, or protonate and dissociate to give nitrogen dioxide (NO_2) and the hydroxyl radical ($OH^\bullet$), which are strong oxidants [8]. Peroxynitrite reacts with protein and non-protein thiols, with tyrosine residues, with unsaturated fatty acids, with DNA, with NO and with a variety of other molecules [8]. Addition of peroxynitrite to mitochondria causes extensive protein modification and cross-linking, and lipid peroxidation [94], so there are multiple effects which are difficult to disentangle.

Addition of peroxynitrite to mitochondria causes irreversible inhibition of respiration at a number of sites [2,95–97] (see Figs. 1 and 2). Note, however, that quite high levels of peroxynitrite (100–500 μM) are required to induce this inhibition, and most of the added peroxynitrite spontaneously decomposes to nitrate with a half-life of about 1 s. Also the 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 reaction of peroxynitrite with these substances [40], and may also be suppressed by many common buffer components such as HEPES [94]. 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 [96] or by adding SIN-1, which is a donor of both NO and superoxide [40]. However, this has some disadvantages as peroxynitrite can react with both NO and superoxide to produce unknown products.

Radi et al. [95] showed that addition of peroxynitrite to isolated mitochondria caused inhibition of complex V (the ATP synthetase, IC_{50} 0.6 mM), complex II (succinate dehydrogenase, IC_{50} 0.9 mM) and complex I (NADH-ubiquinone oxidoreductase, IC_{50}

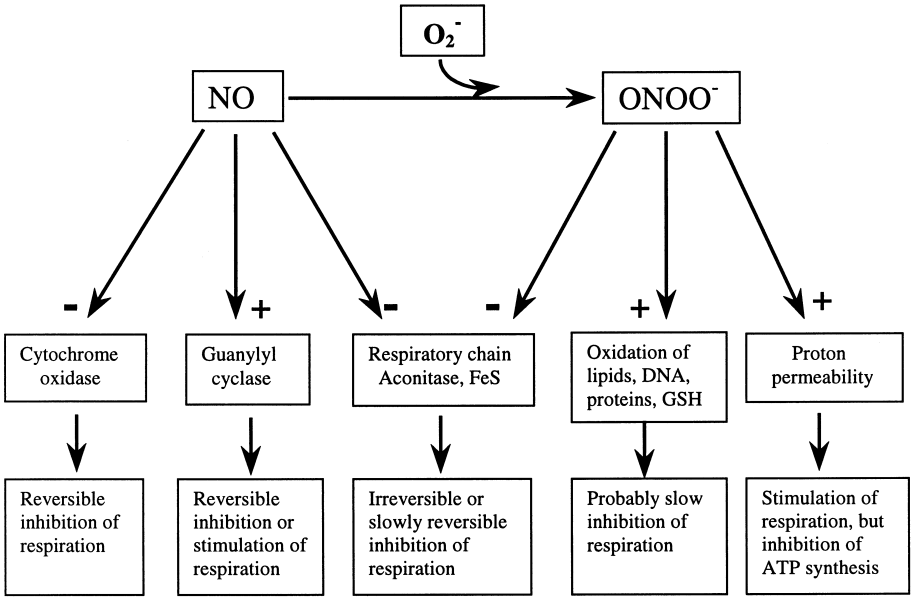

Fig. 2. The actions of NO and peroxynitrite, and their consequences for mitochondrial respiration.

1.5 mM), but relatively little inhibition of complex IV (cytochrome oxidase, $IC_{50} > 2$ mM) [95,96]. 0.5 mM peroxynitrite 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-dependent respiration [95]. Although these are very high levels of peroxynitrite, Radi et al. [95] showed that 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 amount of peroxynitrite. In similar experiments, Lizasoian et al. [40] found that peroxynitrite irreversibly inhibited mitochondrial respiration on NADH or succinate with half-inhibition by 200 μM peroxynitrite, but caused little inhibition of respiration on cytochrome oxidase substrates (ascorbate and TMPD). The mechanisms of peroxynitrite-induced inhibition of complexes I, II, and V are unclear. Hydroxyl radical scavengers do not prevent the inhibition [95], while peroxynitrite anion scavengers, such as thiols, urate and sugars do protect [40,96], suggesting that peroxynitrite itself causes the damage. They might involve modification of protein thiols, or destruction of the iron-sulphur centres in complexes I and II. Peroxynitrite-induced inhibition of succinate dehydrogenase and fumarate reductase in *Trypanosoma cruzi* was reversed by dithiothreitol, suggesting that inhibition was due to nitrosylation of a critical protein thiol [98]. Addition of peroxynitrite to isolated cytochrome oxidase apparently causes inhibition (M.A. Sharpe, C.E. Cooper, personal communication). Bolanos et al. [97] reported that 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. And Brookes et al. [99] reported that this inhibition was also accompanied by an increased proton leak causing mitochondrial uncoupling, which was prevented by lipid soluble antioxidant, Trolox, while Gadelha et al. [94] found a general increase in permeability to all ions, causing mitochondrial swelling, which was partially prevented by DTT. Peroxynitrite can also oxidise reduced (ferro) cytochrome *c* to oxidised (ferri) cytochrome *c* at a rapid rate [100], but this is unlikely to have a significant effect on respiration.

Peroxynitrite (but not NO) can inhibit isolated aconitase, an essential component of the Krebs cycle, and this may cause an inhibition of mitochondrial respiration depending on which substrates are being oxidised and whether aconitase limits respiration [101–103]. The inhibition of isolated aconitase can

be reversed by adding iron and thiols such as dithiothreitol or glutathione, and appears to be due to removal of the α-Fe of the iron-sulphur complex [102]. Only very high levels of NO (>100 μM NO in the absence of oxygen) cause a small reversible inhibition, but nitrosothiols cause a direct inhibition of aconitase not mediated by NO [102]. However, the relative importance of NO and peroxynitrite in inhibiting aconitase remains controversial [104].

Addition of peroxynitrite to mitochondria also causes opening of the permeability transition pore (PTP) (see below [94,105–108]), and opening of this pore causes loss of cytochrome *c* [109–111], and this might contribute to peroxynitrite-induced inhibition of respiration. Pore opening is also likely to uncouple the mitochondria by increasing the proton permeability. Peroxynitrite also caused rapid oxidation of mitochondrial NAD(P)H [107,108] by unknown mechanisms (possibly direct oxidation), followed by hydrolysis of NAD by a cyclosporin-inhibitable mechanism [107]. The NAD(P)H oxidation might be related to the fact that peroxynitrite also rapidly oxidises mitochondrial glutathione [108].

7. NO and peroxynitrite effects on mitochondrial superoxide and hydrogen peroxide production and opening of the permeability transition pore

Poderoso et al. [36] 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 thus it was suggested that the inhibition of complex III caused the superoxide and hydrogen peroxide production [36]. The submitochondrial particles also caused rapid breakdown of added NO, which was prevented by added superoxide dismutase [36], suggesting that the NO-induced superoxide production would result in peroxynitrite production. Radi et al. [95] reported that peroxynitrite addition resulted in greatly increased hydrogen peroxide production by mitochondria (half-maximal stimulation at about 1.5 mM peroxynitrite). Poderoso et al. [37] reported that perfusion of isolated heart with NO resulted in hydrogen peroxide appearing in the perfusate, although this did not appear until 20 min after the perfusion with NO was stopped. They also found that perfusion with bradykinin (which resulted in endogenous NO production) caused a delayed burst of hydrogen peroxide production, which they attributed to the NO interaction with mitochondrial complex III [37].

The NO-induced superoxide and hydrogen peroxide production by mitochondria might result from either: (a) NO interaction with complex III as proposed by Poderoso et al. [36,37], (b) NO inhibition of cytochrome oxidase causing reduction of the respiratory chain, which is known to enhance superoxide production [112], or (c) NO conversion to peroxynitrite, which enhances hydrogen peroxide production [95]. 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 oxidase) into an irreversible inhibition (due to peroxynitrite inhibition of other complexes) (see Fig. 1). However, it should be noted that when NO was added to mitochondria, no peroxynitrite formation was detectable [105], and continuous exposure of mitochondria to 1 μM NO for over 15 min results in no detectable irreversible inhibition of respiration once the NO has been removed [34]. On the other hand it is possible that the continuous production of peroxynitrite, at levels that are too low to detect, can still eventually cause significant inhibition. Peroxynitrite also inhibits the mitochondrial Mn-superoxide dismutase by nitration [113], thus potentially preventing the breakdown of mitochondrial-generated superoxide, and thus favouring further generation of peroxynitrite in mitochondria. The NO-induced hydrogen peroxide production might also be important in converting a reversible inhibition into an irreversible inhibition, as NO rapidly and reversibly inhibits catalase by binding to the haem group of catalase (K_i 0.2 μM NO) [114]. NO does not immediately inhibit glutathione peroxidase (the other main route for hydrogen peroxide breakdown), but it does inhibit 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 [115]. Thus NO may

not only increase hydrogen peroxide production, but also inhibit breakdown.

Addition of peroxynitrite to mitochondria also causes opening of the permeability transition pore (PTP) [94,105–108]. The PTP is a pore in the outer and inner mitochondrial membrane, the opening of which is induced by high calcium and oxidants, and which is specifically inhibited by the immunosuppressant cyclosporin A [2]. PTP is important pathologically because it appears to be a central event in many forms of necrosis and apoptosis [2,3]. However, the molecular nature of this pore is unclear, and the important relation of pore formation to mitochondrial calcium transport is controversial [24]. Packer and Murphy [105–107] reported that peroxynitrite caused calcium efflux, mitochondrial swelling, and depolarisation in liver mitochondria loaded with high calcium, and these changes were inhibited by cyclosporin A [105,106]. Schweizer and Richter [107] obtained similar results, but found that if calcium cycling was prevented there was no mitochondrial depolarisation or general change in permeability, although peroxynitrite induced cyclosporin-sensitive calcium efflux. They interpreted this to mean that peroxynitrite specifically activates a calcium efflux pathway by oxidising critical membrane thiols, and the subsequent calcium cycling can give rise to a general permeability increase [107]. Scarlett et al. [108] found that peroxynitrite rapidly oxidised mitochondrial glutathione to a variety of products, and this led to pore opening, but this permeability transition could be prevented by either dithiothreitol or respiratory substrates that maintained the mitochondrial NAD(P)H in a reduced state. Balakirev et al. [116] found that NO itself could both induce PTP and inhibit PTP; the former effect was suggested to be due to secondary formation of superoxide or peroxynitrite, and the latter effect was suggested to be due NO inhibition of cytochrome oxidase and subsequent membrane depolarisation.

Gadelha et al. [94] found that, in the absence of calcium and the absence of buffer components such as HEPES that scavenge peroxynitrite, peroxynitrite addition to mitochondria still caused mitochondrial swelling and depolarisation, but this was not sensitive to cyclosporin A, but rather associated with oxidation of mitochondrial lipids and cross-linking of mitochondrial proteins. Brookes et al. [99] also observed that peroxynitrite increased the mitochondrial proton permeability, possibly by peroxidation of mitochondrial phospholipids.

8. Exogenous or endogenous NO-induced mitochondrial damage and cell death in cells and tissues

There is a large literature on 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 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, ischaemic, and neurodegenerative pathologies, as well as in host defence against pathogens; and mitochondrial inhibition and damage is one of several means by which NO exerts its cytostatic and cytotoxic effects.

Macrophages, activated with cytokines and endotoxin to express iNOS, can cause cytostasis and/or cytotoxicity of tumour and microbial cells; and this activity is important in non-specific host defence. Isolated macrophages or macrophage-derived cells lines have been co-cultured with tumour 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 since inhibition of NOS or scavenging of NO prevents this cytotoxic activity [6,7,9,10]. And the NO-dependent activity is associated with a characteristic set of metabolic changes in both the target tumour cells and the macrophages, in particular inhibitions of mitochondrial aconitase, complex I, complex II, and nuclear DNA synthesis, and the loss of intracellular iron [6,7,9–14]. These changes are reproduced by incubating the cells anaerobically with NO [7,10], thus it was suggested that the changes are caused by NO displacing iron from the iron-sulphur centres of aco-

nitase, complex I, complex II, and ribonucleotide reductase (a rate-limiting enzyme in DNA synthesis) [7]. Tumour cells co-cultured with activated macrophages showed that some mitochondrial iron-sulphur proteins were inhibited before others, with aconitase being inhibited first ($t_{1/2}$ 4 h), followed by complex I ($t_{1/2}$ 8 h), then complex II ($t_{1/2}$ 14 h), whereas complex III was not inhibited [12].

The inhibition of mitochondrial iron-sulphur proteins in activated macrophages and target cells is accompanied by the appearance of an EPR signal at $g = 2.035$ indicating the presence of a complex between NO and iron, probably of the $[Fe(NO)_2(SR)_2]$ type [117–120]. This complex can be formed in vitro from [2Fe-2S] or [4Fe-4S] clusters reacting with NO, and a wide range of iron-sulphur nitrosyl complexes can give rise to the EPR $g = 2.035$ signal [121]. And both the appearance of this signal and loss of aconitase activity are associated with NO production in a range of different cells, leading to the suggestion that the EPR signal derives from iron released from mitochondrial iron-sulphur centres by NO (see [104,120]). However, relatively little of the iron in cells is present within iron-sulphur centres, and NO treatment of bacteria [122], macrophage extracts [123], and brain nerve terminals [124] gives rise to the EPR $g = 2.035$ signal without any damage to the EPR-visible iron-sulphur centres. Thus, the EPR signal in cells may derive from ferritin or other cytosolic pools of iron (see [104,120]), and is not necessarily a marker for damage to mitochondrial iron-sulphur centres. Mitochondria exposed to NO donors give rise to the EPR $g = 2.035$ signal, but this signal can be completely removed by subsequently incubating the mitochondria with erythrocytes, suggesting that the NO can rapidly dissociate from the iron-nitrosyl complexes [125].

The findings with isolated mitochondria and proteins (see above) that aconitase, complex I and complex II are not apparently inhibited by NO whereas they are inhibited by peroxynitrite, raise the possibility that NO and iNOS induced damage to mitochondria in cells is mediated by peroxynitrite, not NO [95,96,102]. However, this important point is not fully settled as high levels of NO can partially inhibit complex II [80] and aconitase [102].

Szabo et al. [126,127] measured a mitochondrial activity in a macrophage cell line (J774) by assaying the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan in the cells. This assay actually reflects the activity of a number of different dehydrogenases, particularly succinate dehydrogenase [128], and is not a measurement of mitochondrial respiration as such. Szabo et al. [126,127] found that incubating macrophages with either NO donors, a peroxynitrite generator (SIN-1), peroxynitrite, or endotoxin/interferon-γ (to induce iNOS) caused a partial inhibition of this dehydrogenase activity in the cells. Urate (a peroxynitrite scavenger) partially protected the dehydrogenase activity from inactivation by peroxynitrite but not by NO donors, and moderately protected the dehydrogenase activity from inactivation by endotoxin/interferon [126]. Mn(III)tetrakis(4-benzoic acid) porphyrin (MnTBAP, a superoxide and peroxynitrite scavenger) strongly protected the dehydrogenase activity from inactivation by peroxynitrite, moderately protected it against NO donors and endotoxin/interferon [127]. These results suggest that some of the dehydrogenase inhibition by iNOS induction is due to peroxynitrite and some is not [127]; however, it is not clear whether partial inhibition of succinate dehydrogenase would have any significant effect on cellular respiration. Szabo et al. [129] further showed that induction of iNOS in these macrophages caused the oxidation of a large number of mitochondrial and nuclear proteins (detected by reaction with 2,4-dinitrophenylhydrazine) 24 h (but not 12 h) after addition of endotoxin/interferon. This protein oxidation was prevented by a NOS inhibitor, and partially prevented by MnTBAP, suggesting that peroxynitrite may be partially responsible for the oxidation [129]. However, macrophages tend to die after 24 h of iNOS induction, so it is possible that the oxidation might be a consequence, rather than a cause, of cell death.

We recently re-examined the cause of irreversible respiratory inhibition in macrophages treated with NO donors (DETA-NONOate) for several hours [130]. Irreversible inhibition of respiration 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, and surprisingly both the inhibition of respiration and of complex I could be

completely reversed by either light or thiol reducing reagents, such as DTT [130]. This suggests that the inhibition was due to direct nitrosylation of thiols in complex I, which could be reversed by light or reducing agents.

In a variety of other cells types induction of iNOS causes an irreversible inhibition of mitochondrial respiration, usually associated with an inhibition of aconitase, complex I and/or complex II. Thus in hepatocytes, iNOS induction has been associated with inhibition of mitochondrial aconitase and respiration on citrate, malate, and succinate [131,132], and a fall in cellular ATP content [133]. Pre-treatment 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 [131]. The NO-induced inhibition of mitochondrial aconitase and complexes was largely reversible by incubating the cells in the absence of NO [131]. Exposure of hepatocytes to NO donors in the presence of oxygen causes immediate depolarisation 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 oxidase and opening of the mitochondrial PTP [57]. Hepatocytes incubated or co-cultured with activated macrophages or Kupffer cells (resident liver macrophages) show inhibition of aconitase and mitochondrial respiration on all substrates and the appearance of EPR-visible iron-nitrosyl complexes [120,125]. However, induction of iNOS in the Kupffer cells in vivo, causing a dramatic increase in NO production, did not lead to any irreversible inhibition of aconitase or mitochondrial respiration or the appearance of mitochondrial iron-nitrosyl complexes, although it did cause some liver necrosis [125]. This important observation suggests that in vivo the scavenging of NO by oxyhaemoglobin in blood is sufficient to prevent or reverse the NO inactivation of mitochondrial iron-sulphur centres [125].

In fibroblasts, endotoxin/interferon-γ activation of iNOS expression caused inhibition of malate- and succinate-dependent respiration by an L-arginine-dependent process [65]. Similarly in vascular smooth muscle cells, iNOS expression caused NO-dependent inhibition of pyruvate, succinate, and ascorbate/TMPD-dependent respiration (measured after permeabilisation of the cells with digitonin) [66]. This respiratory inhibition could be prevented by uric acid (a peroxynitrite scavenger) and by inhibitors of poly(ADP) ribosyltransferase (PARS), suggesting that respiratory inhibition was in part mediated by peroxynitrite activation of PARS [134]. NO-dependent inhibition of cellular respiration has also been observed in isolated perfused lung [135]. In cardiac myocytes cytokine-induced expression of iNOS caused inhibition of succinate dehydrogenase, as measured by the MTT assay, and this inhibition was reversed by inhibiting iNOS (with NMA) or by removing the cytokines [67].

In brain, endotoxin/interferon-induced iNOS expression in cultured astrocytes or microglia caused an 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, and all these effects were preventable by inhibiting NOS [136]. The irreversible inhibition of cytochrome oxidase was partially prevented by adding high concentrations of Trolox, a vitamin E analogue, suggesting that this inhibition was mediated via lipid peroxidation, perhaps by peroxynitrite [137]. However, addition of up to 2 mM peroxynitrite to astrocytes caused no inhibition of mitochondrial complexes, whereas addition of peroxynitrite to cultured neurones caused inhibition of complexes II–III and complex IV, but not complex I, measured 24 h after peroxynitrite addition, and accompanied by cell death [97]. Induction of iNOS in astrocytes also causes a potent and reversible inhibition of the oxygen consumption of the cells, due to the acute NO inhibition of cytochrome oxidase in competition with oxygen, and this inhibition can be immediately reversed by inhibiting iNOS or binding the NO with haemoglobin [58].

Brief exposure to low levels of NO is cytotoxic to neurones, by mechanisms that are not entirely clear, but may involve inhibition of mitochondrial respiration (see [23,138] for reviews). NO potently and reversibly inhibits the mitochondrial respiration of isolated nerve terminals [15], and causes release of the neurotoxic neurotransmitter glutamate from these terminals due to the NO inhibition of cytochrome oxidase [56]. The neurotoxicity of NO may partially,

and depending on conditions, be due to the NO-induced glutamate release, which is then toxic to the neurones via NMDA receptors [139]. This toxicity may be enhanced when NO is inhibiting mitochondrial respiration and oxidative phosphorylation, as (a) this will cause depolarisation of the plasma membrane and thus dramatically increase the sensitivity of the NMDA receptor to glutamate, and (b) this will depolarise the mitochondrial membrane and thus decrease the buffering of cytosolic calcium. Expression of iNOS in mixed astrocytic-neuronal cocultures causes delayed neuronal death (see [23,138]) and inhibition of mitochondrial complexes II–III and complex IV, but not complex I and citrate synthase in the neurones [140]. The same pattern of mitochondrial inhibition is caused by adding peroxynitrite to neurones, suggesting that the iNOS expression in astrocytes/microglia may inhibit the respiration of co-cultured neurones via peroxynitrite [97].

NO can induce apoptosis in some cell types, including macrophages, thymocytes, T cells, myeloid cells, and neurones, and this may be mediated by the effects of NO on mitochondrial respiration [141,142]. Addition of NO to these cells causes inhibition of respiration, depolarisation of the mitochondrial membrane potential, release of mitochondrial apoptogenic factors into the cytosol, activation of caspases, and thus apoptosis [142]. 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 [143–146]. The decision between apoptosis and necrosis may partly depend on the ATP level, since a large drop in ATP level can block apoptosis but promote necrosis [147]. The level of cellular thiols, such as glutathione, is also important since thiols scavenge peroxynitrite and can reverse many of the effects of NO and peroxynitrite, partly by re-reducing oxidised and nitrosylated protein thiols [148]. The presence of glucose and rate of glycolysis can also be important, 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 [7,66]. The level of oxygen is also important in determining if and how cells die in response to NO [149], as this affects the sensitivity of cytochrome oxidase to NO, and the rate of breakdown of NO to NO_2 and N_2O_3.

9. Discussion

The effects of NO and of peroxynitrite on mitochondria are clearly distinct, and should be distinguished (see Table 1). NO causes a reversible and relatively specific inhibition of cytochrome oxidase. Although high levels of NO for long periods can cause other effects, which may be mediated by the reversible nitrosylation of protein thiols and the perhaps removal of iron from iron-sulphur centres. Peroxynitrite by contrast potentially oxidises most things in the mitochondria, causing 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 membrane. Unfortunately the effects of NO_2 and N_2O_3 on mitochondria are unclear at the moment. It has been thought that their rate of production would be low, but the finding that the reaction between NO and oxygen is much faster within bilayer membranes [54] suggests that NO_2 and N_2O_3 may be more important in NO-induced toxicity than previously thought. The effects of the nitroxyl ion and nitrosothiols on mitochondria are also relatively unclear.

Can the NO inhibition of cytochrome oxidase mediate a physiological regulation of respiration by NO? The evidence from in vitro systems is clear that NO could play such a role. However, in vivo NO rapidly reacts with myoglobin and haemoglobin so that the NO level may never reach a level in vivo at which cytochrome oxidase is inhibited. On the other hand this argument equally applies to the NO activation of the soluble guanylyl cyclase, which certainly does occur in vivo. The sensitivity of isolated guanylyl cyclase to NO (dissociation constant of 250 nM NO) [150] is rather similar to that of cytochrome oxidase, but the rate of deactivation of the activated cyclase is poorly characterised [151], and the sensitivity to NO of cGMP-dependent process in tissue seems to be one or two orders of magnitude higher than cytochrome oxidase at high oxygen concentrations, but may be more similar at low oxygen concentrations [150,151]. The levels of NO

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

Component affected	Effector	Nature of effect	Reference
Cytochrome oxidase	NO	50–500 nM causes reversible inhibition competitive with oxygen	[15–17,34–42]
Cytochrome oxidase	Peroxynitrite	Irreversible inhibition (>2 mM)	[95–97]
Cytochrome oxidase	iNOS	Inhibition in astrocytes	[97,136,137]
Cytochrome c^{3+}	NO	Reversible binding, K_d 20 μM	[82]
Cytochrome c^{2+}	NO	Reaction producing cyt c^{3+} and NO_2^-	[82]
Cytochrome c^{2+}	Peroxynitrite	Reaction producing cyt c^{3+}	[100]
Complex III	NO	Reversible inhibition	[13,80]
Ubiquinol	NO	Reaction	[79]
Complex II	NO	2mM NO removes iron-sulphur centre	[80]
Complex II	Peroxynitrite	Irreversible inhibition	[95,96,98]
Complex II	iNOS	Macrophages and target tumour cells	[6,7,9–14]
Complex II	NO in cells	Inhibition after incubation with high levels	[7,10,131]
Complex I	iNOS	Macrophages and target tumour cells	[6,7,9–14]
Complex I	NO in cells	Inhibition after incubation with high levels	[7,10,131]
Complex I	Peroxynitrite	Inhibition	[95,96]
NADH	Peroxynitrite	Oxidation and hydrolysis	[107]
Aconitase	NO	Partial inhibition by high levels	[102]
Aconitase	Peroxynitrite	Inhibition reversible by DTT plus iron	[101–103]
Aconitase	iNOS	Inhibition	[7,10]
Aconitase	NO in cells	Inhibition	[7,10,131]
Creatine kinase	NO	Inhibition reversible by DTT	[84,85]
Creatine kinase	Peroxynitrite	Irreversible inhibition	[86]
ATP synthase	Peroxynitrite	Inhibition	[95,96]
Proton and ion leaks	Peroxynitrite	Increase	[94,99]
PTP	Peroxynitrite	Open pore and calcium efflux	[94,105–108]

present in vivo are unclear at present, partly because the only measurements so far made in vivo (10 nM–5 μM NO) are with Malinski's NO-selective electrode, which apparently measures the NO within the membrane bilayers where the NO concentration is higher [152]. However, the use of NO synthase inhibitors in vivo has shown that inhibiting the basal NO production causes a marked stimulation of tissue and whole body oxygen consumption, consistent with a basal inhibition of tissue respiration by NO. On the other hand we do not yet know that this stimulation is not mediated by cGMP or some other mechanism, rather than by cytochrome oxidase. Further in vivo experiments are required to determine whether NO really is a direct physiological regulator of oxidative phosphorylation.

If NO is a physiological regulator of cytochrome oxidase, then what is the physiological rationale for such a regulation of energy metabolism? Poderoso et al. [36] suggested that the capillary endothelium produced a gradient of NO within the surrounding tissue, resulting in an inhibition of respiration close to the endothelium, which functioned to redistribute the oxygen supplied to cells further away from the vessel wall. However, this reasoning does not take into account the competition between oxygen and NO at cytochrome oxidase, which results in a greater inhibition of the oxidase in the cells with the lowest oxygen level, i.e. the cells that most need the oxygen. Shen et al. [19] suggested that capillary endothelium-derived NO regulated muscle contractility, via its inhibition of muscle respiration and ATP production, but why such regulation should occur teleologically was not discussed. Perhaps, since endothelial NO regulates oxygen supply, it makes sense to also regulate oxygen consumption, and the competitive inhibition of cytochrome oxidase will have the effect of transferring control over energy metabolism from ATP utilisation to oxygen supply. Inhibition of mitochondrial respiration will raise tissue oxygen levels and thus compete with NO to limit further inhibition of respiration. The NO inhibition of respiration will

also increase glycolysis, and thus may also function to regulate the relative ATP supply from glycolysis and mitochondria. Resting skeletal muscle is an important thermogenic organ due to continuing mitochondrial respiration. As NO synthase inhibitors increase the oxygen consumption of resting skeletal muscle in vivo, the NO may be functioning to regulate thermogenesis. The finding that mitochondria from all tissues are capable of generating NO and regulating their own respiration by this means raises new possibilities, but until we know how this mitochondrial NO synthase is regulated it is difficult to speculate what factors may regulate respiration by this means or for what purpose. If the mtNOS is of the eNOS type then it may be acutely regulated by calcium and phosphorylation, whereas if it is of the iNOS type it may only be regulated by gene expression.

Brown [18] pointed out that the NO inhibition of cytochrome oxidase increased the apparent K_m of mitochondrial respiration for oxygen into the physiological range of oxygen concentrations, thus potentially making respiration sensitive to oxygen supply. In a variety of tissues, organisms and conditions respiration can become very sensitive to the oxygen level [153,154], and the competition between NO and oxygen at cytochrome oxidase might play a role in this sensitivity. However, there is no evidence for this as yet, and it should be noted that the apparent K_m of NO synthases for oxygen is at least an order of magnitude higher than that of cytochrome oxidase (in the absence of NO), so that at moderately low oxygen levels NO synthase might be unable to produce sufficient NO to inhibit cytochrome oxidase.

The NO inhibition of cytochrome oxidase might play a role in the cytotoxicity of iNOS expressing cells towards bacteria, other pathogens, tumour cells, host cells, and iNOS expressing cells themselves. Certainly iNOS expressing cells in culture can reversibly inhibit cellular respiration by this means. And the reversible inhibition of respiration might be converted into an irreversible inhibition of respiration by a variety of means, including: (a) necrosis caused by ATP depletion, (b) peroxynitrite production due to increased superoxide production by mitochondria, (c) glutamate release in brain, (d) opening of the permeability transition pore, and (e) calcium release from mitochondria.

The mechanisms by which NO kills (or protects) cells are still far from clear. A variety of mechanisms appear to be involved, which differ in different conditions. But one of those mechanisms involves the generation of peroxynitrite from NO, and the subsequent damage to mitochondria, which may induce necrosis or apoptosis. Peroxynitrite also damages non-mitochondrial targets, including nuclear DNA, and these targets may be more important for cell death in some conditions. And the definitive evidence that peroxynitrite is mediating mitochondrial damage in variety of pathologies and NO cytotoxicity is still lacking. NO can kill cells by mechanisms independent of peroxynitrite, but peroxynitrite can kill cells and damage mitochondria, and in many models of NO-induced cytotoxicity peroxynitrite seems to be the most likely villain.

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