Review

Regulation of mitochondrial respiration by nitric oxide inhibition of cytochrome *c* oxidase

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Abstract

Nitric oxide (NO) and its derivatives inhibit mitochondrial respiration by a variety of means. Nanomolar concentrations of NO immediately, specifically and reversibly inhibit cytochrome oxidase in competition with oxygen, in isolated cytochrome oxidase, mitochondria, nerve terminals, cultured cells and tissues. 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. Isolated mitochondria, cultured cells, isolated tissues and animals in vivo display respiratory inhibition by endogenously produced NO from constitutive isoforms of NO synthase (NOS), which may be largely mediated by NO inhibition of cytochrome oxidase. Cultured cells expressing the inducible isoform of NOS (iNOS) can acutely and reversibly inhibit their own cellular respiration and that of co-incubated cells due to NO inhibition of cytochrome oxidase, but after longer-term incubation result in irreversible inhibition of cellular respiration due to NO or its derivatives. Thus the NO inhibition of cytochrome oxidase may be involved in the physiological and/or pathological regulation of respiration rate, and its affinity for oxygen. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

This paper reviews what is known about nitric oxide (NO) regulation of mitochondrial respiration via reversible NO inhibition of cytochrome *c* oxidase. A number of other reviews of this or related topics have appeared recently [1–5]. NO has a variety of effects on mitochondria that need to be distinguished: (a) at low concentrations, for short periods, NO specifically and reversibly inhibits cytochrome oxidase, but (b) higher levels of NO, more prolonged exposure to NO, or conversion of NO to one of its more reactive oxides results in irreversible inhibition of respiration and other damage to mitochondria. This review concentrates on the former, reversible effects of NO, but in order to distinguish these effects, and to understand the literature historically, I will first briefly outline the biology of NO and the irreversible effects of NO on respiration.

In eukaryotic cells NO is mainly produced by the enzyme nitric oxide synthase (NOS), of which there are three known isoforms: nNOS (neuronal NOS, type I NOS), iNOS (inducible NOS, type II NOS) and eNOS (endothelial NOS, type III NOS) [6,7]. NOS uses substrates arginine, NADPH and oxygen, and produces citrulline, NADP and NO. nNOS and eNOS are constitutively expressed in endothelium, platelets, some neurons and at lower levels a variety

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of other cells. nNOS and eNOS are acutely activated by calcium/calmodulin, alternatively they may be activated or inhibited by phosphorylation via various protein kinases [6]. iNOS is not normally expressed, but its expression is induced by cytokines (such as interferon-γ, IL-1β, and TNF-α), endotoxin (LPS), and/or oxidative stress. Once iNOS is expressed it is continuously active and is not dependent on elevated calcium, so that it can produce higher levels of NO and for a much longer period than eNOS or nNOS. eNOS and nNOS are transiently activated, and are involved in regulating physiological functions, whereas iNOS is expressed and is continuously active during inflammation, where it is involved in host-defence against pathogens. There is some evidence that mitochondria may also contain NOS (mtNOS) [8,9], but the nature of this mtNOS and its relation to other NOS isoforms is still unclear. NO may also be generated non-enzymatically from nitrite at low pH in the presence of reductants, but again it is unclear how significant this pathway is [10]. The main sources of NO and their activators are summarised in Fig. 1.

Once NO is produced it may react with a number of molecules (Fig. 2). NO reacts rapidly with high affinity to ferrous iron (Fe^{2+}), either free iron, iron within iron–sulphur centres, or iron within haemproteins, particularly those where the haem has a free ligand position [11]. The major physiological actions of NO (such as relaxation of smooth muscle, neurotransmission, and inhibition of platelet aggregation and adhesion) are mediated by NO binding to the ferrous haem iron of soluble guanylate cyclase, causing a stimulation of cGMP production [12]. The main route by which NO is broken down in the body is thought to be the reaction of NO with haemoglobin in blood [13]. But NO can also bind to and inhibit other haemproteins with a free ligand posi-

Fig. 2. Main reactions of NO. NO may reversible bind to ferrous iron within haemproteins, such as guanylate cyclase, haemoglobin and cytochrome oxidase. At high concentrations NO may react with oxygen to produce NO_2 and N_2O_3. NO reacts at the diffusion limited rate with superoxide to produce peroxynitrite. NO, after oxidation to NO^+ or peroxynitrite, can react with thiols to produce nitrosothiols.

tion, such as cytochrome oxidase and catalase [11]. NO also reacts directly with oxygen to produce NO_2 and N_2O_3, which are strong oxidants, but this reaction is thought to be relatively slow at physiological levels of NO [14]. NO reacts at the diffusion limited rate with superoxide (O_2^-) to produce another strong oxidant peroxynitrite ($ONOO^-$), and peroxynitrite has in the past been blamed for much of the cytotoxicity of NO [14]. NO can also react indirectly with thiols (e.g. glutathione or protein cysteine residues) to produce S-nitrosothiols (RSNO) [15]. However, NO itself does not directly react with thiols, but requires conversion to NO^+ or an NO^+ donor, such as peroxynitrite, NO_2 or N_2O_3 [15]. Nitrosothiols can directly transfer the NO^+ group to other thiols, and may regulate protein functions by this means [15].

2. Irreversible inhibition of respiration by NO and its derivatives

In the 1980's it was shown that activated macrophages could kill tumour cells, and this NO-dependent activity was 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 and complex II, and the loss of intracellular iron [16–20]. These changes were reproduced by incubating the cells anaerobically with high levels of NO [17,19], and it was suggested that the changes were caused by NO displacing iron from the iron–sulphur centres of aconitase, complex I and complex II [17]. High levels of NO (about 1 mM) can indeed reversibly or irreversibly damage the mitochondrial iron–sulphur centres

Fig. 1. Sources of NO. In mammalian cells NO may be derived from different isoforms of NO synthase (nNOS, iNOS or eNOS), or possibly mitochondrial NOS (mtNOS), and in acid conditions reductants may reduce nitrite to NO.
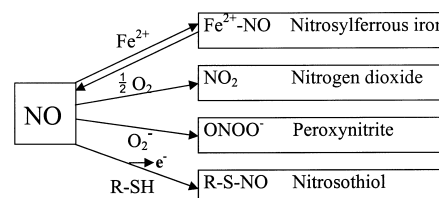
[21,22] but whether this is responsible for the irreversible inhibition of cellular respiration by NO is less clear.

NO can rapidly react with superoxide to produce peroxynitrite ($ONOO^-$), and peroxynitrite can cause irreversible inhibition of mitochondrial respiration and damage to a variety of mitochondrial components via oxidising reactions. Peroxynitrite inhibits or damages mitochondrial complexes I and II, the ATP synthase, aconitase, creatine kinase, the mitochondrial membrane, mitochondrial DNA, superoxide dismutase, and induces mitochondrial swelling, uncoupling, depolarisation, calcium release and permeability transition (see [1,23–25]). Peroxynitrite or high levels of NO (> 1 μM) also irreversibly inhibit cytochrome oxidase and raise its K_m for oxygen [26]. Thus peroxynitrite has been blamed for much of the pathology and cell death induced by NO; however, it still remains unclear whether NO-induced cell death or irreversible inhibition of mitochondrial respiration is partly mediated by peroxynitrite.

The cause of irreversible respiratory inhibition was recently re-examined in cultured cells treated with the NO donor DETA-NONOate (2,2′-(hydroxynitroso-hydrazino) bis-ethanamine, also known as NOC-18) for several hours [27,28]. Irreversible inhibition of respiration occurred after a delay of several hours, at a time when cellular glutathione levels were depleted, and was mainly due to the inhibition of complex I. Surprisingly both the inhibition of respiration and of complex I could be completely reversed by light, dithiothreitol or glutathione methyl ester, suggesting that the inhibition was due to nitrosylation of essential thiols in complex I [27–29]. We have recently found that the *S*-nitrosothiols: *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) and *S*-nitrosoglutathione (SNOG) can inhibit complex I in mitochondria even in conditions when there is very little NO release from the nitrosothiols (in the dark and presence of copper chelators), and this inhibition can be reversed by light and reduced thiols [30]. This suggests the possibility that complex I activity may be regulated by reversible *S*-nitrosylation. Creatine kinase [31,32] and the mitochondrial permeability transition pore [33] also appear to be sensitive to nitrosothiols.

It is possible that brain cells respond to NO in a somewhat different manner than other cells. Complex I in isolated brain mitochondria appears to be relatively resistant to the direct actions of peroxynitrite [34]. However, this sensitivity may be critically dependent on the concentration of mitochondria used and the presence of substrates, as peroxynitrite may be scavenged by a wide variety of chemicals that may be present in media [25]. Induction of iNOS in astrocytes or brief exposure of cultured neurons to peroxynitrite did not result in any loss of complex I activity [35]. In contrast, when neurons were exposed to SNAP for 24 h, there was marked loss of complex I activity, in conjunction with decreased activity of complexes II, III and IV [36]. Under such conditions there was a marked loss of neuronal glutathione (GSH). When compared to neurons, astrocytes appear relatively resistant to peroxynitrite [34]. But when astrocytes were depleted of GSH, they became more sensitive to added peroxynitrite, accompanied by marked inhibition of complex I following peroxynitrite exposure [37]. These findings, together with those in the previous paragraph, suggest that NO/peroxynitrite/nitrosothiol-induced complex I inhibition only occurs after cellular GSH depletion.

Other irreversible or pathological effects of NO may be mediated by NO conversion to the strong oxidants NO_2 or N_2O_3 (see [1]), but we need to distinguish all such irreversible effects of high levels of NO from the acute, reversible inhibition of cytochrome oxidase by low levels of NO, reviewed below.
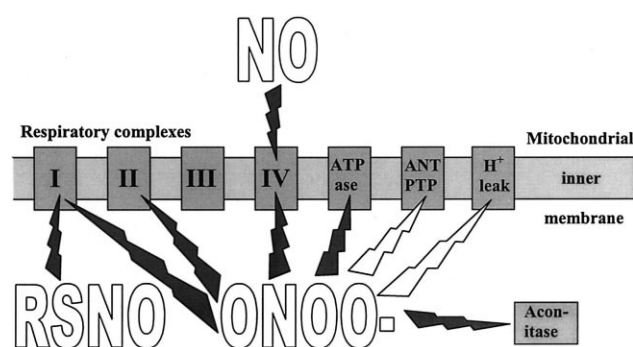

Fig. 3. Main actions of nitric oxide (NO), peroxynitrite ($ONOO^-$) and nitrosothiols (RSNO) on mitochondria. NO specifically and reversibly inhibits cytochrome oxidase (complex IV); nitrosothiols inactivate complex I; whereas peroxynitrite inhibits multiple respiratory complexes and aconitase, and activates the proton leak and permeability transition pore (ANT-PTP), which may contribute to NO-induced cell death. Inhibitions are indicated by dark arrows/bolts, while light ones indicate activations.

The major actions of NO and its derivatives on mitochondria are summarised in Fig. 3.

3. 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 is essential for virtually all energy production in cells [38]. 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. The oxidase contains two haems (cyt a and cyt a_3) and two copper centres (Cu_A and Cu_B). Oxygen binds to the reduced form of a binuclear centre consisting of cytochrome a_3 (Fe^{2+}) and Cu_B (Cu^{1+}) within the complex, and this constitutes the oxygen binding site and catalytic centre of the oxidase [38].

NO binds to the oxygen binding site of cytochrome oxidase [39–41]. 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 [42–44], although an earlier report strongly suggested this [45]. We [42] 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. We [42] 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. We found [42] 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) (Fig. 4). We subsequently [46] reported that similar levels of NO reversibly inhibited respiration in isolated heart mitochon-
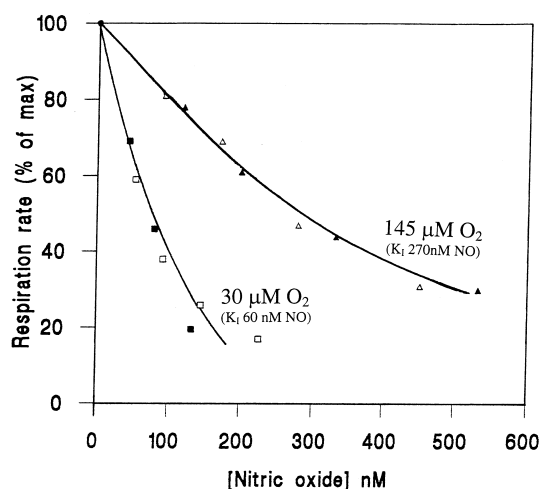

Fig. 4. Dependence on NO concentration of the oxygen consumption of synaptosomes at two different oxygen concentrations. Synaptosomes (nerve terminals isolated from the brain, containing many mitochondria) were incubated in a sealed, stirred vessel with NO at two different oxygen levels, with an oxygen and NO electrode. Adapted from Brown and Cooper [42].

dria due to the inhibition of cytochrome oxidase, but oxygen consumption was markedly less sensitive to NO in state 4 than in state 3, probably because cytochrome oxidase has less control over (is less rate limiting for) mitochondrial respiration rate in state 4. Cassina and Radi [23] reported that somewhat higher levels of NO were required to inhibit oxygen consumption in heart mitochondria, but they did not actually measure the levels of NO. Poderoso et al. [47,48] 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). Others [49–52] reported that addition of about 1–4 µM NO to isolated mitochondria caused inhibition of oxygen consumption and ATP synthesis, which was greater at lower oxygen levels and largely reversible. Cleeter et al. [43] showed that an NO donor caused a reversible inhibition of oxygen consumption in isolated skeletal muscle mitochondria due to inhibition of cytochrome oxidase. Lizasoain et al. [24] 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 [44] 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.

NO also potently inhibits the cytochrome oxidase activity of *Paracoccus denitrifans* [45] and *Escherichia coli* [53], and this might contribute to bacterial toxicity of NO. Cytochrome oxidase within higher plant mitochondria is also inhibited by NO [54].

In isolated cytochrome oxidase (from beef heart) Torres et al. [55] and Giuffre et al. [56] 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-Cu_B 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 us [42], but again the actual levels of NO present were not measured. Light can dissociate NO from the oxidase [40], and we have recently found that light can reverse the NO inhibition of mitochondrial respiration [30]. 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}) [41], and NO dissociates from the oxidase slowly (although relatively rapidly compared to other haemproteins, $k = 0.1–0.01$ M^{-1} s^{-1}) [56], indicating a very high affinity of the reduced enzyme for NO (K_d about 1 nM). But oxygen also binds rapidly to the Fe^{2+} of cyt a_3 ($k = 10^8$ M^{-1} s^{-1}) [38], and at high ratios of oxygen to NO (normally > 1000:1) it appears anomalous that NO can inhibit cytochrome oxidase so rapidly (less than 1 s) by binding to cyt a_3 alone. Thus Torres et al. [55] proposed that during turnover, NO preferentially binds to the reduced form of Cu_B (Cu^{1+}), 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 Cu_B^{2+}, forming Cu^{1+}–NO^+, the nitrosonium (NO^+) may then hydrate to give nitrite, which may inhibit the oxidase [57]. Nitrite is a poor inhibitor of the oxidase when added externally (requiring high millimolar concentrations), but if formed within the binuclear centre it can be an effective inhibitor, because diffusion out of (or into) the binuclear centre pocket is slow. The binding of NO to Cu^{1+} or Cu^{2+} might be the major pathway for rapid inhibition of the oxidase. However, Giuffre et al. [56] believe that the rapid onset of inhibition may be explained in terms of rapid binding of NO to the Fe^{2+} of the partially reduced binuclear centre. Oxygen only binds to the fully reduced binuclear centre (Fe^{2+}–Cu^{1+}), not to the partially reduced forms (Fe^{2+}–Cu^{2+} or Fe^{3+}–Cu^{1+}), thus if NO could bind to either of the latter, it would gain a kinetic advantage over oxygen in binding and inhibiting the binuclear centre. Whatever the mechanism of rapid inhibition by NO, it is clear that only the reaction of NO with the (partially or totally) reduced binuclear centre can account for the competition between oxygen and NO, which is characteristic of this form of respiratory inhibition by NO. Reactions of NO with cytochrome oxidase have previously been studied by Brudvig et al. [39].

Because NO competes with oxygen at cytochrome oxidase, NO acts to increase the apparent K_m of respiration for oxygen [42], and this led me [58] to suggest that NO may be a physiological regulator of the oxygen sensitivity of respiration in tissues. Koivisto et al. [59] 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. Koivisto et al. [59] suggested that this might be caused by two molecules of NO competing with one molecule of O_2; however, Stubauer et al. [60] showed that only one molecule of NO bound to cytochrome oxidase (at least in the reduced form). It is not clear why Koivisto et al. [59] found a different oxygen dependence of the NO inhibition of respiration than that found by Brown and Cooper [42] (reproduced in Fig. 4). But it may well be a consequence of the difficulty of measuring inhibited respiration rates at low oxygen levels in an ordinary Clarke oxygen electrode. Since the average level of oxygen in mammalian tissues is about 30 μM O_2, the results of Koivisto et al. [59] 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.

NO can be produced by three different isoforms of NO synthase (eNOS, nNOS and iNOS), located in the cytosol or cell membranes, but NO synthase activity or proteins binding to NOS-specific antibodies have been found in isolated mitochondria, indicating either that one of the three known isoforms can bind to or be transported into mitochondria, or that there is a new mitochondrial isoform (mtNOS) [8,61–63]. Giulivi et al. [64] 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 submitochondrial particles, suggesting that the NOS was located on the inner mitochondrial membrane. Tatoyan and Giulivi [9] 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 [65] 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. Ghafourifar and Richter [8,66] found that mitochondrial NO production was stimulated by calcium, and the NO so produced could decrease mitochondrial respiration, membrane potential and pH gradient, and regulate mitochondrial pH and calcium buffering. They further found that calcium stimulation of mtNOS may contribute to calcium activation of cytochrome c release and subsequent apoptosis [67]. Thus, the high activity of mtNOS, location on the inner mitochon-

drial membrane, and demonstration that it can regulate mitochondrial respiration suggests that it may in fact function to regulate mitochondrial respiration physiologically. However, doubts still remain as to the nature and significance of mtNOS. Recently it was suggested that mitochondria may produce NO by another mechanism, due to reduction of nitrite to NO by ubisemiquinone in the cytochrome bc_1 complex [68].

NO may also be consumed by mitochondria due to (a) reaction with mitochondrial superoxide to produce peroxynitrite, (b) reaction with ubiquinol to produce ubiquinone and NO^-, which may then further react to give N_2O or peroxynitrite, and possibly (c) reaction at cytochrome oxidase to produce N_2O and nitrite [46,69,70]. The production of peroxynitrite by mitochondria might contribute to irreversible inhibition of respiration.

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 [42,71], hepatocytes [72], astrocytes [73], pancreatic β-cells [74], kidney tubules [75], neurons [76] and ascites tumour cells [51]. This respiratory inhibition was largely reversed when the NO was removed, although there was also an irreversible component to the inhibition in some cases (e.g. [36]). 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 [72], and pancreatic β-cells [74], and a decrease in mitochondrial membrane potential and cellular ATP in ascites tumour cells [51] and neurons [76]. NO-induced respiratory inhibition was also accompanied by insulin release by β-cells and intact pancreatic islets [74] and glutamate release by nerve terminals [71].

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 the next section.

4. 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 [77]. (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 [17,73]. We used primary cultures of astrocytes, isolated from brain, activated to express iNOS by interferon-γ and endotoxin and found to produce up to 1 μM NO, and this endogenous NO caused potent inhibition of cellular respiration at cytochrome oxidase, which was rapidly reversed by either removing the NO with oxyhaemoglobin or by inhibiting NO synthase [73]. We also found that a macrophage cell line (J774) activated to express iNOS caused rapid and strong inhibition of the cellular respiration of co-incubated cells (L929 fibroblasts or non-activated macrophages) via the reversible NO inhibition of cytochrome oxidase [78]. Furthermore we have 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, transient inhibition of cellular respiration due to the inhibition of cytochrome oxidase [79]. Also the background, unstimulated release of NO was sufficient to partially inhibit endothelial cell respiration, and strongly increases the apparent K_m of respiration for oxygen, so that inhibition of NOS caused a stimulation of respiration and decrease in K_m for oxygen.

Miles et al. [80] 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. Similarly Sarti et al. [81] reported that addition of NOS inhibitors to neuroblastoma cells rapidly increased the mitochondrial membrane potential within the cells, while NO donors had the opposite effect, again suggesting a constitutive inhibition of cytochrome oxidase by NO within cells. Shen et al. [82] 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. In heart muscle slices incubated in vitro Xie et al. [83] found 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. A similar bradykinin-induced inhibition of respiration was found in the isolated left ventricle wall of mice, but was absent in the wall from eNOS knockout mice [84]. These results suggest that endothelial agonists cause activation of eNOS and the NO produced diffuses from the endothelium to myocytes, where the NO directly inhibits the mitochondrial respiratory chain or substrate supply. However, the reversibility and oxygen dependence of this inhibition were not measured, so we can not be sure that it was due to NO inhibition of cytochrome oxidase. Poderoso et al. [48] 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 was not measured, so the mechanism of the inhibition is unclear.

In vivo in conscious dogs Shen et al. [85] 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. Shen et al. [82] found that NMA also increased the oxygen consumption in hind limb skeletal muscle of conscious dogs by 55%. And Laycock et al. [86] 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. They also found that bradykinin or NO donors decreased the oxygen consumption renal slices in vitro. King et al. [87] 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. [88] found that NMA caused a 28% increase in heart oxygen consumption of exercising dogs. Loke et al. [89] found that administration of blood-free haemoglobin conjugates (that efficiently scavenge NO) to conscious dogs doubled heart oxygen consumption, while decreasing fatty acid oxidation and increasing glucose and lactate oxidation.

All of the above results are consistent with the interpretation that basal constitutive NO release by capillary endothelium or by NOS within muscle cells tonically inhibits tissue respiration. However, it can not be ruled out that this inhibition is mediated via cGMP and/or ATP consumption, as cGMP can weakly inhibit oxygen consumption of muscle slices [82], and can inhibit isolated myocyte contraction [77] and oxygen consumption [90].

In contrast to the concept that cGMP can inhibit muscle and heart oxygen consumption, it has been reported that cGMP and NO donors can stimulate the oxidation of glucose, pyruvate, palmitate and leucine in isolated soleus muscle via cGMP-depen-

dent mechanism [91–93]. This stimulation was suggested to be involved in the physiological stimulation of glucose oxidation by muscle. However, in heart in vivo endogenous NO appears to stimulate fatty acid oxidation and inhibit carbohydrate oxidation, while inhibiting oxygen consumption [89,94]. The mechanism by which NO changes substrate utilisation is unknown. Clearly more work needs to be done to untangle the multiple effects of NO/cGMP on respiration within tissues and in vivo.

5. Discussion

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 guanylate cyclase, which certainly does occur in vivo. The levels of NO present in vivo are unclear at present, partly because the only measurements so far made in vivo (10 nM–5 μM NO) are with a porphyrinic-based NO-selective electrode (rather than the more commonly used Clarke-type polarographic electrode), which apparently measures the NO within the membrane bilayers where the NO concentration is higher [95]. 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.

I have previously pointed out that the NO inhibition of cytochrome oxidase increases the apparent K_m of mitochondrial respiration for oxygen into the physiological range of oxygen concentrations, thus potentially making respiration sensitive to oxygen supply [58]. In a variety of tissues, organisms and
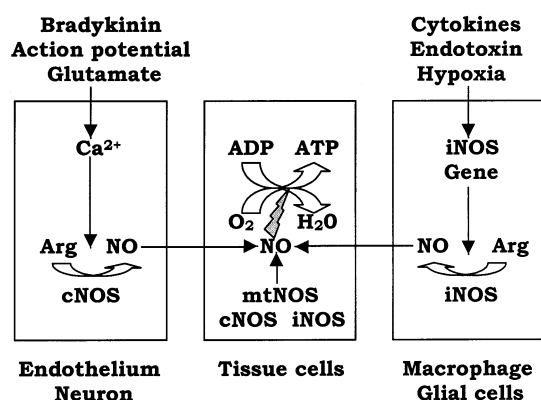
Fig. 5. Scheme of how NO, derived from either constitutive forms of NOS (cNOS), mitochondrial NOS (mtNOS), or inducible NOS (iNOS), may regulate mitochondrial respiration.

conditions respiration can become very sensitive to the oxygen level [96,97], and the competition between NO and oxygen at cytochrome oxidase might play a role in this sensitivity. However, there is no direct evidence for this in vivo, and it should be noted that the apparent K_m of NO synthase 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. Note, however, that during ischaemia, tissue pH decreases and may promote the reduction of nitrite to NO in the absence of oxygen [10], thus potentially causing potent inhibition of cytochrome oxidase at low oxygen levels.

Fig. 5 summarises the potential role of NO in regulating mitochondrial respiration. Constitutive forms of NOS present in endothelium (eNOS), neurons (nNOS) and other cells such as myocytes (eNOS and nNOS) may produce NO acutely and transiently when stimulated by appropriate agonists, resulting in a transient inhibition of respiration in these and surrounding cells, which might function either to regulate thermogenesis, ATP-dependent functions or oxygen sensitivity. If mtNOS exists it may play similar roles. iNOS, when expressed in macrophages and many other cells in inflammatory conditions, produces sustained, high levels of NO, which is likely to strongly inhibit respiration in these and surrounding cells, potentially causing cytotoxicity in those cells that are unable to survive on glycolytic ATP production.

The physiological/teleological role of NO regula-

tion of mitochondrial respiration is unclear, and the following is speculation. NO from iNOS and from constitutive NOS (eNOS and nNOS) probably have different roles, as iNOS produces higher levels for a sustained period. The main role of iNOS expression during inflammation appears to be to suppress proliferation and induce cytostasis of pathogens, host cells, and tumour cells. The NO-induced inhibition of respiration within these cells may well contribute to the cytostasis and cytotoxicity, and there is some evidence for this role in vitro, but not so far in vivo. In terms of constitutive NOS we need to distinguish between several possible modes of regulation: (a) transcellular/paracrine regulation of one cell by another, (b) intracellular/autocrine regulation of a cell's respiration by NO produced within that cell, and (c) intramitochondrial regulation by NO produced within the mitochondria. Transcellular respiration might act to regulate respiration in surrounding cells due to NO production by: (a) endothelium or (b) neurons. Endothelial NO might regulate respiration in (a) vascular smooth muscle in blood vessels, or (b) tissue cells regulated by capillary endothelium. The latter might allow hormones or other humoral factors to regulate tissue respiration. In the peripheral nervous system NO synthase is expressed in some parasympathetic neurons and might enable activity in this system to regulate tissue respiration.

Acknowledgements

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