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Review

Nitric oxide and mitochondrial respiration in the heart

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Abstract

Nitric oxide (NO) inhibits the mitochondrial respiratory chain, resulting in inhibition of ATP production, increased oxidant production and increased susceptibility to cell death. NO reversibly binds to the oxygen binding site of cytochrome oxidase, reacting either with the oxidised copper to give inhibitory nitrite, or with the reduced haem, resulting in reversible inhibition in competition with oxygen. Because of this competition, NO may sensitise tissues to hypoxia. NO, or derivative N_2O_3 or S-nitrosothiols, may inactivate complex I by S-nitrosation. Peroxynitrite ($ONOO^-$) inhibits mitochondrial respiration at multiple sites, and also causes mitochondrial permeability transition. Inhibition of mitochondrial respiration by NO and its derivatives stimulates production of reactive oxygen and nitrogen species by mitochondria, which have signalling roles in the heart, but may also contribute to cell death. In the heart, NO is produced by endothelial NO synthase (eNOS) in endothelium and caveolae of cardiomyocytes, by neuronal NO synthase (nNOS) in sarcoplasmic reticulum and possibly mitochondria, and under pathological situations by inducible NO synthase (iNOS) in the sarcoplasm. Haemoglobin and myoglobin may have multiple roles in determining oxygen and NO gradients within the heart, which may remove NO at high oxygen, but possibly supply it at low oxygen. Stimulating or inhibiting NOS in the heart has been found to cause small changes in heart oxygen consumption *in vivo*; however, it is still unclear whether these changes are due to direct NO inhibition of mitochondrial respiration or indirect actions of NO. NO inhibition of mitochondrial respiration is likely to be more important in the heart during hypoxia and/or pathologies where iNOS is expressed.

Keywords: Oxygen radicals; Nitric oxide; Mitochondria; Cell death; Permeability transition; Heart; Cytochrome oxidase; Oxygen consumption; Energy metabolism; Inflammation

1. Introduction

Mitochondrial respiration and its regulation by nitric oxide are important in the heart for several reasons. Firstly, mitochondria generate almost all the ATP required for muscle contraction in the heart, so that inhibition of mitochondrial respiration results in an inhibition of contractility. Secondly, inhibition of mitochondrial respiration stimulates mitochondrial production of reactive oxygen species (ROS) which regulates signal transduction pathways within the heart. Thirdly, inhibition of mitochondrial respiration can cause necrosis or apoptosis within the heart. In this review we outline the interactions between NO and its derivative reactive nitrogen species (RNS) with the mitochondrial respiratory chain, and we discuss what impact this has on mitochondrial respiration with particular regard to the heart. Other pathophysiological aspects of the effects of NO on mitochondrial functions have been reviewed elsewhere [1–3].

2. NO hits the mitochondria

NO interacts with the mitochondrial respiratory chain by different means: (A) NO itself causes rapid, selective, potent, but reversible inhibition of cytochrome oxidase, and (B) reactive nitrogen species (RNS, which include $ONOO^-$ (peroxynitrite), NO_2, N_2O_3 and S-nitrosothiols) cause slow, non-

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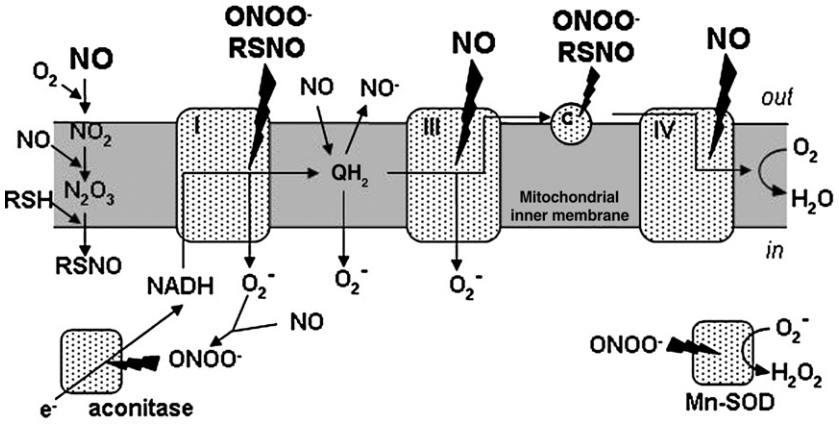

Fig. 1. Interactions of NO, reactive nitrogen species (RNS), reactive oxygen species and the mitochondrial respiratory chain. NO reacts with oxygen in the mitochondrial membrane to produce NO_2 and N_2O_3, and the latter can react with thiols (RSH) to produce S-nitrosothiols (RSNO). NO and RNS inhibit respiratory complexes I, III and IV resulting in superoxide production that reacts with NO to give peroxynitrite ($ONOO^-$), which further inhibits the respiratory chain, aconitase and the Mn-superoxide dismutase (Mn-SOD). Inhibitions are indicated by a lightning bolt.

selective, weak, but irreversible (or slowly reversible) inhibition of many mitochondrial components (see Fig. 1).

NO causes rapid and reversible inhibition of cytochrome oxidase at nanomolar levels of NO [4,5], so that NO is potentially a physiological regulator of respiration. NO reversibly inhibits cytochrome oxidase apparently by two different means involving NO binding to two different components of the oxygen binding site, which in both cases blocks oxygen binding. The oxygen-binding site consists of two metals, the iron of haem a_3 and the copper of the Cu_B centre, and oxygen binds between them (and is rapidly reduced by them) when both metals are reduced (a_3^{2+} and Cu^+). NO can either (1) bind to reduced cytochrome a_3 to give cytochrome a_3^{2+}-NO, or (2) NO can bind and reduce oxidised Cu_B^{2+} to give Cu_B^+–NO^+, and the NO^+ can rapidly hydrate to give nitrite (NO_2^-) [6–8]. Both forms of inhibition are rapid and reversible, due to debinding of NO in (1) and debinding of nitrite in (2). The first form of inhibition is competitive with oxygen and reversible by light whereas the second is not, and these characteristics may be used to distinguish between them [6]. It seems that at least in vitro both forms of inhibition may occur simultaneously, but the first form is favoured at high levels of cytochrome reduction and low oxygen, whereas the second form is favoured by the opposite conditions [6,8]. However, the inhibition observed in cells in response to NO or expression of inducible NO synthase (iNOS or NOS2) appears to be largely competitive with oxygen [4,9,10] and reversible by light [11], suggesting that inhibition due to reversible binding to haem a_3^{2+} may be more important in cells.

Endogenously produced NO may tonically inhibit respiration at cytochrome oxidase in some cells [9,12,13]. Inhibition is generally in competition with oxygen, so that NO can dramatically increase the apparent K_M of respiration for oxygen [4,9,14,15]. In synaptosomes half-inhibition of respiration occurred at 250 nM NO when the oxygen level was about 150 μM (roughly the arterial level), but the K_i was about 60 nM NO at 30 μM O_2 (a median tissue level) [4]. According to this data then the presence of 60 nM NO would raise the apparent K_M of respiration for oxygen from below 1 μM to 30 μM O_2: well into the physiological range. A variety of cells (including macrophages, microglia, astrocytes, endothelial cells and aorta) when inflammatory activated to express iNOS, have been shown to produce sufficient NO to not only inhibit their own respiration but also that of surrounding cells via reversible NO inhibition of cytochrome oxidase [10,14–16].

Apart from acute, direct and reversible inhibition of cytochrome oxidase, NO/RNS can cause persistent inhibition of the complexes of the respiratory chain due to protein modifications. Peroxynitrite has relatively little effect on the V_{max} of cytochrome oxidase when added to mitochondria at levels that inhibit the other complexes [17,18]. However, it does have various damaging effects on isolated cytochrome oxidase, including particularly increasing the K_M for oxygen [19]. High concentrations (>1 μM) of NO (possibly via NO_2 or N_2O_3) can also induce an irreversible rise in K_M for oxygen both in isolated cytochrome oxidase or in cells treated with NO [19,20]. Recently it has been shown that prolonged exposure of porcine pulmonary artery endothelial cells to NO donor DETA/NO resulted in a gradual, persistent inhibition of complex IV concomitant with a reduction in ratios of mitochondrial GSH and GSSG [21]. The inhibition of the enzyme was due to S-nitrosation of subunit II. This subunit contains two cysteine residues and when these cysteines of complex IV were mutated, S-nitrosation of complex IV by NO was attenuated [21].

The other mitochondrial respiratory chain complexes (complexes I, II and III) are less potently, but more persistently, inhibited by NO or RNS, and this type of inhibition may be relevant to pathology where iNOS is induced. In particular, complex I (the NADH-ubiquinone oxidoreductase) is inactivated in a variety of different ways [22], including probably S-nitrosation (addition of a NO^+ group to

a thiol residue) [11,23,24] and nitration (addition of a NO_2^+ group, usually to a tyrosine residue) [25–27]. Complex II (the succinate-ubiquinone oxidoreducatse) is relatively insensitive to NO or RNS, but the iron–sulphur centres may be damaged by high levels of RNS [17,28]. Complex III (the cytochrome bc_1 complex) may be reversibly inhibited by high levels of NO, but the mechanism is unclear [29,30].

Cytochrome *c*, a small soluble protein of the respiratory chain, contains four tyrosine residues and cysteine which has been shown to undergo nitration [31,32] and S-nitrosation [33]. There is some controversy concerning redox properties of nitrated cytochrome *c* [31,32], however, various groups of investigators agree that function of nitrated or S-nitrosated cytochrome *c* as an electron carrier to support oxygen consumption by mitochondria is inhibited [31–34].

NO and RNS have a variety of other effects on mitochondria that may impact on energy production. Peroxynitrite can inhibit mitochondrial aconitase, an enzyme of the Kreb's cycle [35–37]. Mitochondrial creatine kinase, which aids the export of ATP from mitochondria in muscle and nerves, is inhibited by S-nitrosothiols, probably by transnitrosation of a critical thiol [38,39]. Inhibition of NOS *in vivo* also causes an acute change from fatty acid to glucose oxidation (increased 4 fold) in heart, possibly resulting from activation of the pyruvate dehydrogenase complex [40]. NO can stimulate mitochondrial biogenesis via cGMP and increased expression of transcription factors PGC-1α, NRF-1 and mtTFA, resulting in increased respiratory chain complexes [41].

NO and RNS can also stimulate ROS (reactive oxygen species) and RNS production by mitochondria, which may be important signals in heart, mediating for example ischaemic preconditioning [42]. At high levels, NO can react directly with oxygen in the mitochondrial bilayer to give NO_2 and N_2O_3 [43]. At moderate levels NO can acutely increase O_2^- and H_2O_2 production by inhibiting mitochondrial respiration, while at higher levels it inhibits H_2O_2 production by scavenging the precursor superoxide, resulting in peroxynitrite production [12,29,44]. NO may also apparently react with ubiquinol (QH_2) to produce NO^- (which may react with O_2 to produce $ONOO^-$) and ubisemiquinone ($QH^\bullet$) (part of which may react with O_2 to produce O_2^-) [45]. S-nitrosothiol-inactivation of complex I can also reversibly increase ROS production from complex I several fold [23,46]. NO and RNS inhibition of respiration may result in local peroxynitrite production (due to local superoxide production) causing irreversible inhibition of respiration and further oxidant production — a vicious cycle that might contribute to cell death [25,44]. In addition to stimulating H_2O_2 production, NO or RNS can also inhibit catalase, deplete cellular glutathione and inhibit glutathione peroxidase, thus increasing H_2O_2 levels in cells [12,44,47] (see Fig. 1.).

In vitro, peroxynitrite and S-nitrosothiols can cause direct permeabilisation of mitochondrial membranes, and this effect is inhibited by cyclosporin A, indicating involvement of the mitochondrial permeability transition pore (MPT) in the permeabilisation event [48–50]. Opening of MPT pore has two important implications: (1) MPT causes mitochondrial depolarization and subsequent inhibition of ATP synthesis which may lead to cellular ATP depletion and necrotic cell death; and (2) opening of MPT pore can cause the release of several proteins from mitochondria, including cytochrome *c*, which may lead to activation of caspases and apoptosis. NO itself may promote MPT by inhibiting respiration and thus lowering the membrane potential which favours MPT [51]. However, in the absence of a membrane potential, mitochondria can not take up calcium to trigger MPT, and this may be the dominant effect of NO [52]. NO may affect MPT indirectly through (a) activation of soluble guanylate cyclase leading to activation of PKG which may phosphorylate some, yet unidentified components of MPT resulting in lower probability of MPT opening [53,54], (b) modulation of cellular calcium homeostasis [1]; or (c) stimulation of mitochondrial ROS production.

3. Gradients of NO and oxygen

NO is produced by different isoforms of NO synthase in different locations within and outside of cells, and NO is consumed (particularly by haemoglobin and myoglobin) in different locations (see Fig. 2). This has the potential to produce gradients of NO concentration within and between cells. Similarly, oxygen is supplied (particularly by haemoglobin and myoglobin) in different locations than those in which it is consumed (mainly mitochondria). Thus the O_2 activity in the arterial system is about 150 μM, whereas the median O_2 availability in heart sarcoplasm is about 4 μM (estimated from myoglobin saturation), and the oxygen concentration at cytochrome oxidase itself may be substantially lower than this because the oxidase is the main sink for oxygen [55]. However, it should be noted that both NO and O_2 diffuse rapidly and pass straight through membranes, so that it is difficult to build up significant gradients on the scale

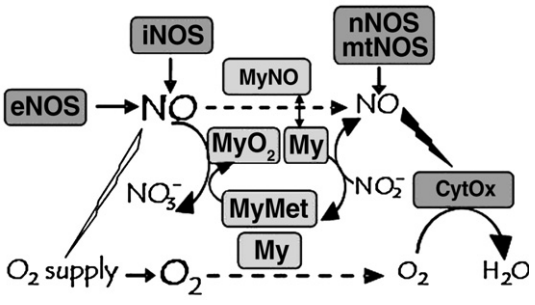

Fig. 2. NO inhibition of cytochrome oxidase in heart. In normoxia NO is scavenged by oxymyoglobin (MyO_2) to produce nitrate and metmyoglobin (MyMet), so that little reaches cytochrome oxidase in mitochondria. In hypoxia, NO is reversibly bound to deoxymyoglobin (My), but NO may be produced by nitrite reacting with deoxymyoglobin to give metmyoglobin. Thus in hypoxia more NO may reach cytochrome oxidase, and also may more effectively inhibit it due to less competition with oxygen. Mitochondrial-located NOS (mtNOS) and nNOS may produce NO locally to inhibit cytochrome oxidase, while NO from eNOS in endothelium and caveolae may vasodilate to supply oxygen.

of cells unless they are produced and consumed very rapidly [56]. The gradients of NO and O_2 may interact in several ways: (a) NO inhibits the main consumer of oxygen (cytochrome oxidase) in a manner that depends on the NO and O_2 concentration at the mitochondria, (b) NO is a major regulator of oxygen supply via vasodilation of vascular smooth muscle, (c) the K_M of NO synthases for O_2 is potentially within the physiological range within the heart, and (d) haemoglobin and myoglobin produce NO at low oxygen and consume NO at high oxygen, and NO oxidation of the globins inactivates their ability to transport oxygen (and NO).

NO binds to deoxymyoglobin and deoxyhaemoglobin at the diffusion limited rate, and dissociation occurs on a time scale of hours [57], thus deoxymyoglobin may act as a temporal and spatial buffer of NO levels in the myocytes (particularly at low oxygen levels when myoglobin is deoxygenated). NO reacts with oxymyoglobin and oxyhaemoglobin at the diffusion limited rate to produce nitrate and metmyoglobin and methaemoglobin [57]. This is probably the main metabolic fate of NO in the heart [58], however there is also evidence that most NO in the heart is oxidized by cytochrome oxidase to nitrite [59,60]. Oxidation of NO by oxymyoglobin will potentially increase the gradients of NO concentration within myocytes as well as decreasing the overall concentration (particularly at high oxygen levels when myoglobin is present as oxymyoglobin). Deoxymyoglobin and deoxyhaemoglobin react with nitrite to produce NO [61], thus at low oxygen levels NO may be produced independent of NO synthases.

Neuronal NO synthase (nNOS or NOS1) colocalizes with the ryanodine receptor in the sarcoplasmic reticulum, and activation of nNOS increases cardiac contractility [62,63]. In contrast, endothelial NO synthase (eNOS or NOS3) coupled to the β_3 adrenergic receptor in caveolae, inhibits L-type Ca^{2+} channels and, thus, inhibits β-AR-mediated increases in myocardial contractility [62]. And there is evidence that the NO generated by eNOS and nNOS is confined to its location of production, so that stimulation of eNOS or nNOS has different effects on the heart [63]. As the sarcoplasmic reticulum is closely connected to the mitochondria, it is possible that NO from nNOS located in sarcoplasmic reticulum is more likely to reach the mitochondria than NO generated by eNOS in the caveolae of cardiomyocytes or eNOS in the endothelium. eNOS in the endothelium may be more likely to affect mitochondria within the endothelial cells themselves.

nNOS may also be found bound to mitochondria as the so-called mitochondrial NO synthase (mtNOS) [64], but levels of mtNOS in heart are apparently low [65], and its existence is controversial as several groups can not find it at all [66], including in heart [67]. It is possible that the apparent presence of nNOS in mitochondria is due to contamination with sarcoplasmic reticulum [68]. However, others suggest that 60% of heart cytosolic NO production comes from the mitochondria, and the rate of production has an exponential dependence on mitochondrial membrane potential [69]. Arginase is an enzyme that removes the substrate for NO synthase, and arginase II has been found to be localized to mitochondria within cardiomyocytes, such that specific inhibition of arginase II increases nNOS activity (in mitochondria or sarcoplasmic reticulum), resulting in an inhibition of contractility [70].

Oxygen is a substrate for all NO synthases with apparent K_M values of 4, 130 and 350 μM O_2 for eNOS, iNOS and nNOS respectively [71], so that nNOS and iNOS may be substrate limited by oxygen. iNOS is not normally expressed in the healthy heart, but its expression is dramatically increased by inflammation and after ischaemia/reperfusion [72,73]. Once expressed in the cytosol of cardiomyocytes, iNOS (NOS2) can produce high, sustained levels of NO, which have the potential both to inhibit mitochondrial respiration and to oxidise myoglobin, so decreasing oxygen supply to the mitochondria.

It has been suggested that a potential function of NO inhibition of mitochondrial respiration at cytochrome oxidase is to redistribute oxygen away from tissue close to blood vessels towards tissue further away from vessels (that are potentially hypoxic), i.e. oxygen gradients from vessel into tissue would be made less steep by NO inhibition of oxygen consumption close to the vessel [74]. We believe this is unlikely because NO inhibition of cytochrome oxidase is competitive with oxygen, therefore a flat gradient of NO within tissue would have exactly the opposite effect, i.e. the NO would inhibit oxygen consumption far from the vessel where the oxygen concentration is low, but not inhibit oxygen consumption close to the vessel where the oxygen concentration is high.

NO inhibition of cytochrome oxidase does not require a specific function, but one possible function is to regulate heat production and temperature of tissues. Hyperthermia increases the activity of NO synthases, and the resulting NO will increase blood flow by vasodilation, thus decreasing tissue temperature in heart and other internal organs that produce heat. At high NO levels, mitochondrial respiration will also be inhibited, resulting in an inhibition of heat production (because mitochondria are the main source of heat in all tissues). In isolated cardiomyocytes, it was found that mild heat shock increased the activity of eNOS via HSP90 resulting in NO inhibition of cytochrome oxidase within the cells [75]. Thus, NO may be part of a feedback loop to prevent excessive heating and heat damage of the heart and other tissues, for example during exercise.

4. NO inhibition of respiration in the heart

NO rapidly and reversibly inhibits the oxygen consumption of mitochondria isolated from heart, mainly due to the inhibition of cytochrome oxidase [76]. However, it is unclear whether this occurs *in vivo* because myoglobin and haemoglobin are present in heart at high concentrations and deplete NO rapidly. Knockout of the myoglobin gene in mice increases the sensitivity of cardiac respiration to NO [58,77], suggesting that one function of myoglobin is to reduce or

prevent NO inhibition of mitochondrial respiration [57]. However, several factors make NO inhibition of respiration more feasible than at first seems likely in the presence of myoglobin. Firstly, the median O_2 concentration in sarcoplasm is about 4 μM in heart [55], thus only 9 nM NO or less would be required to half inhibit cytochrome oxidase at this oxygen level. Secondly, nNOS may be found within heart mitochondria [78], and may in fact be bound directly to cytochrome oxidase [79]. Thirdly, at low oxygen levels as occur in the heart close to the mitochondria, most of the myoglobin is present as deoxymyoglobin, which can produce (rather than consume) NO from nitrite [61,80].

Stimulation of perfused rat heart with bradykinin or carbachol (which activate eNOS in the endothelium) causes an NOS-dependent inhibition of oxygen consumption (and increased hydrogen peroxide production), suggesting that NO from endothelial eNOS could reach cytochrome oxidase in cardiomyocytes [12,77]. Similarly, in tissue slices of heart ventricle, it was shown that bradykinin and carbachol inhibited mitochondrial respiration, but not when the eNOS gene was knocked out or NOS inhibited [81]. The inhibition of respiration was increased in heart slices for mice heterozygous for mitochondrial superoxide dismutase, suggesting that mitochondrial superoxide regulates the availability of NO at mitochondria [77]. However, in neither the slices or perfused heart was the reversibility or mechanism of the inhibition characterized, and it remains possible that this inhibition was mediated by cGMP, as cGMP (at high concentrations) has been shown to inhibit the oxygen consumption of isolated heart mitochondria (by a relatively small amount) [13]. Also tissue slices often have a hypoxic core and hyperoxic surface, which complicates the extrapolation of results obtained with slices to the in vivo situation. In contrast to the above authors, Kojic et al. [82] found that stimulation of eNOS with bradykinin in perfused mouse heart caused no change in oxygen consumption (even though NO production was stimulated 5 fold), suggesting that, at least in mouse, NO from eNOS is insufficient to inhibit mitochondrial respiration.

Deoxymyoglobin can generate NO from nitrite, and it has been shown recently that addition of reasonable levels of myoglobin and nitrite to isolated mitochondria inhibits oxygen consumption due to NO inhibition of cytochrome oxidase, and further addition of nitrite to heart homogenates inhibits oxygen consumption by this mechanism [80].

The sensitivity of soluble guanylate cyclase to NO appears to be at least two orders of magnitude higher than that of cytochrome oxidase, which might indicate that the latter is not a significant physiological target for NO [16]. However, inhibition of NOS *in vivo* has been shown to cause substantial increases in organ and whole-body oxygen consumption, apparently not due to changes in blood flow, consistent with a tonic inhibition of mitochondrial respiration *in vivo* [13,83–87]. NOS inhibitors may cause a small increase in cardiac oxygen consumption *in vivo* [88,89], consistent with reversing a tonic NO inhibition of cytochrome oxidase. An NO scavenger (polymerized haemoglobin) was found to double the oxygen consumption of hearts in conscious dogs, and switch oxidation from fatty acids to glucose, independently of any haemodynamic changes [90]. However, it remains unclear whether these changes are mediated by cGMP or a direct effect on cytochrome oxidase, or via changes in ATP consumption or respiratory substrate supply. It is important to repeat these experiments in vivo or in perfused heart, and test whether the changes in oxygen consumption are reversible by inhibiting NOS or by inhibiting soluble guanylate cyclase or by light or by raising the oxygen level.

Inflammation, ischaemia and heart failure result in iNOS expression in heart, and the subsequent depression of contractile activity and oxygen consumption has been blamed on NO inhibition of mitochondrial respiration. Inflammatory cytokines cause iNOS expression in heart and decrease oxygen consumption, at least in part via inhibition of the respiratory chain [72]. Ischaemic preconditioning causes iNOS expression in heart, and recent evidence suggests that this causes a substantial inhibition of oxygen consumption of hearts *in vivo* [73]. Heart failure causes iNOS expression, and a decrease in oxygen consumption at rest and work that is reversed by iNOS inhibitors [88], suggesting that NO from iNOS inhibits cytochrome oxidase causing contractile dysfunction. Inflammatory iNOS expression in other tissues greatly sensitises to hypoxia-induced cell death [14,15], but it is unknown whether this occurs in heart.

However, transgenic mice overexpressing iNOS (100 fold) specifically in the heart had no significant change in contractile or energetic phenotype [91] unless myoglobin was inactivated by carbon monoxide [92]. On the other hand, another group found that iNOS expression in the heart, even at relatively low levels, resulted in a severe contractile phenotype [93].

5. Conclusions

NO interacts with the mitochondrial respiratory chain by reversible and irreversible means. The extent to which NO inhibits respiration in vivo in healthy hearts is probably low due to the presence of myoglobin, but may be greater in the presence of iNOS and/or hypoxia. Important issues that need to be resolved now include the following.

(a) To test whether the observed changes in heart oxygen consumption *in vivo* are mediated by direct inhibition of mitochondrial respiration by NO, or by indirect means.
(b) To determine whether mtNOS or mitochondrial-associated NOSs are real or an artefact, and to determine whether artificially targeting nNOS to the mitochondria causes significant inhibition of mitochondrial respiration *in vivo*.
(c) To determine the local activity of NO and oxygen in vivo at the mitochondria and other locations in the cell (perhaps by targeting sensitive proteins to these locations).

(d) To determine the consequences of pathophysiological levels of iNOS expression in the heart on contractile function, oxygen consumption, temperature, mitochondrial signalling and hypoxia sensitivity.

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