Hypothesis

Nitric oxide regulates mitochondrial respiration and cell functions by inhibiting cytochrome oxidase

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Abstract Nitric oxide (NO) reversibly inhibits mitochondrial respiration by competing with oxygen at cytochrome oxidase. Concentrations of NO measured in a range of biological systems are similar to those shown to inhibit cytochrome oxidase and mitochondrial respiration. Inhibition of NO synthesis results in a stimulation of respiration in a number of systems. It is proposed that NO exerts some of its main physiological and pathological effects on cell functions by inhibiting cytochrome oxidase. Further NO may be a physiological regulator of the affinity of mitochondrial respiration for oxygen, enabling mitochondria to act as sensors of oxygen over the physiological range.

Key words: Nitric oxide; Cytochrome *c* oxidase; Mitochondria; Oxygen sensor; Muscle relaxation; Cytokines

1. Introduction

Nitric oxide (NO) has a number of physiological roles, including: (a) relaxation of smooth muscle; (b) neurotransmission and neuromodulation; (c) inhibition of platelet aggregation and adhesion; and (d) killing of pathogens (reviewed in [1–5]). NO can also be toxic to host cells, and has been implicated in variety of pathological processes. NO is known to be produced by a several isoforms of nitric oxide synthase, but the effector system by which NO exerts its effects on cells is less clear. The prime candidate for NO's effector system has been guanyl cyclase, since NO binds to and stimulates guanyl cyclase and thus might control cell functions via cGMP and the cGMP-dependent protein kinase. However, the evidence that guanyl cyclase mediates responses to NO is relatively indirect. I suggest here that some of the major physiological and pathological effects of NO are mediated via its inhibition of cytochrome oxidase, and thus of cellular ATP production (see Fig. 1).

Nitric oxide has been known to bind to cytochrome oxidase for about 20 years, and has been used as an experimental tool for investigating the spectra and mechanism of cytochrome oxidase [6]. However, its potential as a regulator of respiration was not investigated, as NO was not known to be a physiological mediator until about 5 years ago. Recently it has been found that NO rapidly and reversibly inhibits the steady-state turnover of isolated cytochrome oxidase at sub-micromolar concentrations of NO [7]. The inhibition has also been seen as a reversible inhibition of respiration in isolated submitochondrial particles [8], mitochondria [9], brain nerve terminals [7], cultured astrocytes [10], muscle slices [11,12], and as a reversible decrease in mitochondrial membrane potential in isolated mitochondria [13] and cells [14] (see Table 1). The inhibition of cytochrome oxidase is competitive with oxygen [7], and NO binds with high affinity to the oxygen binding site of cytochrome oxidase when this site is reduced [6].

Inhibition of cytochrome oxidase will cause decreased ATP production, and thus increased cellular levels of ADP, AMP, GDP and P_i. The levels of these metabolites regulate a large range of cellular processes, including muscle contraction, protein synthesis and ion transport [15], and thus inhibitors of cytochrome oxidase potentially regulate these processes. However, strong, long-term inhibition of cytochrome oxidase is toxic to cells which cannot activate glycolytic ATP production sufficiently to supply essential ATP requiring reactions; and indeed continuous high levels of NO are toxic to many cells (see below). On the other hand, NO inhibition of cytochrome oxidase may be self-limiting because: (a) inhibition of cytochrome oxidase, which consumes most of the oxygen in the body, will raise tissue oxygen levels, which will both compete with NO at cytochrome oxidase and promote NO breakdown; and (b) cytochrome oxidase itself may catalyse NO breakdown [6].

Because NO competes with oxygen at cytochrome oxidase, NO raises the apparent K_m of cytochrome oxidase for oxygen, potentially making mitochondrial respiration sensitive to oxygen concentration over the physiological range. A variety of evidence indicates that the apparent K_m (K_a) for oxygen of respiration is much greater in intact tissues and cells (>1 μM) than in isolated mitochondria and isolated cytochrome oxidase (<1 μM) [16,17]. A number of explanations of these findings have been suggested including macroscopic and microscopic diffusion gradients. But another possible explanation is a competitive inhibitor of cytochrome oxidase (e.g. NO) which is present in the intact systems but not the isolated systems. A number of tissues (e.g. the vasculature and carotid body) are able to sense physiological changes in oxygen concentration, but the mechanism of oxygen sensing is unclear. The presence of NO in these tissues would enable cytochrome oxidase to act as an effective oxygen sensor, and I suggest that NO may be an important physiological regulator of mitochondrial respiration, via adjusting the K_m of cytochrome oxidase for oxygen.

2. Quantitative assessment of the feasibility of NO regulation of respiration in vivo

In this section I will try to make some quantitative assessment of: (a) whether an inhibitor of cytochrome oxidase could

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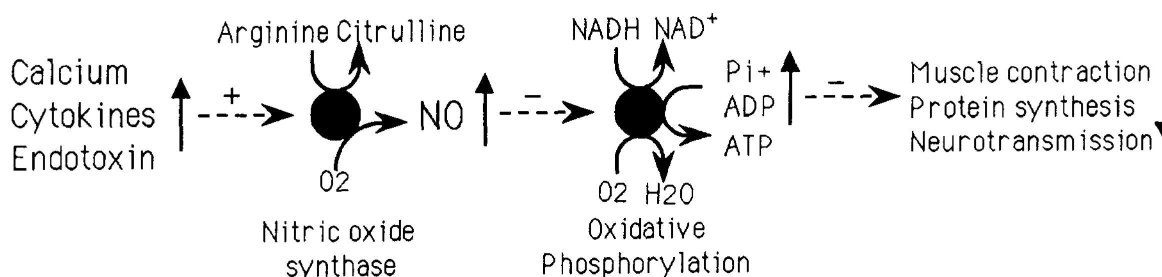

Fig. 1. Scheme illustrating the hypothesis that NO exerts physiological and cytotoxic effects via inhibiting cytochrome oxidase within oxidative phosphorylation, resulting in a rise in cellular ADP and P_i levels, which in turn inhibits many cellular ATP-utilising processes.

regulate mitochondrial respiration and ATP turnover; (b) the levels of NO found to inhibit cytochrome oxidase and respiration; (c) the levels of NO produced in physiological systems; and (d) whether stimulation of NO production does in fact inhibit respiration.

Cytochrome oxidase is the terminal complex of the mitochondrial electron transport chain, and is responsible for virtually all the oxygen consumption of tissues. It is the only component of oxidative phosphorylation known to operate far from equilibrium, and thus has often been considered as a potential site for regulation of mitochondrial ATP synthesis [15]. Cytochrome oxidase has been measured to have significant control over respiration in isolated mitochondria (control coefficient of 0.17 over state 3 respiration in isolated liver mitochondria [18]), indicating that inhibition of cytochrome oxidase will in fact inhibit mitochondrial respiration at least in isolated mitochondria. Inhibitors of cytochrome oxidase or other components of oxidative phosphorylation can cause reversible inhibitions of multiple cell functions, such as muscle contraction, protein synthesis, ion transport, or neurotransmitter release [15,19]. Oxygen concentration appears to limit respiration in some tissues [15–17], suggesting that cytochrome oxidase is also limiting, and thus an agent (such as NO) that decreased the affinity of cytochrome oxidase for oxygen should inhibit respiration.

Are the concentrations of NO found to inhibit cytochrome oxidase and respiration comparable to the concentrations of NO produced in physiological systems? The concentration of NO required to half inhibit respiration in synaptosomes was 270 nM at around 145 μM O_2 (roughly the arterial concentration of oxygen) and 60 nM NO at around 30 μM O_2 (roughly the tissue level of O_2). Similar levels of NO are required to inhibit respiration in isolated cytochrome oxidase and cells (Table 1). Endogenous levels of NO measured in various systems range from 0.01 to 5 μM, with 0.1 to 1.0 μM NO being common after stimulation (Table 2). Thus it would appear that in some of these systems the level of NO is sufficient to cause significant and even substantial inhibition of cytochrome oxidase (depending on the oxygen concentration).

Table 1
Systems where NO has been shown to inhibit respiration

[NO] (μM)	System	Ref.
0.2	Cytochrome oxidase (isolated)	[7]
–	Paracoccus cells and vesicles	[8]
–	Submitochondrial particles	[8]
0.06–0.27	Brain nerve terminals (isolated)	[7]
–	Mitochondria (isolated)	[9]
0.1–0.8	Astrocytes (cultured)	[10]
–	Muscle (isolated)	[11,12]

Exogenous NO was added and levels of NO required to half inhibit respiration are indicated, unless not known (–). Note that inhibition is oxygen dependent.

Is there any direct evidence that stimulation of NO production causes inhibition of respiration, while inhibition of NO production causes stimulation of respiration in a physiological system? In vascular smooth muscle cytokines have been shown to induce NO synthase resulting in inhibition of mitochondrial respiration [28]. In the hindlimb of anaesthetized dogs inhibition of NO synthase resulted in a 40% increase in oxygen consumption of the limb despite a decrease in blood flow [29]. In conscious dogs inhibition of NO synthase resulted in a immediate 25% increase in whole body oxygen consumption, prompting the suggestion that endothelial NO tonically inhibits tissue respiration [30]. In isolated skeletal and heart muscle, bradykinin (which causes NO release from the endothelium) caused inhibition of tissue respiration, and the respiratory inhibition was reversed by inhibiting NO synthase [11,12]. In cultured astrocytes induction of NO synthase with interferon-γ and endotoxin caused the cells to produce 0.4–1.0 μM NO and resulted in a 45% inhibition of respiration at high oxygen concentrations and 80% at low oxygen concentrations [10]. The inhibition was immediately reversed when either NO synthase was inhibited or the NO was bound with haemoglobin. The inhibition was attributed to cytochrome oxidase on the basis of the substrate, oxygen and NO sensitivity [10]. Thus in these systems there is direct evidence that NO is involved in regulating cellular respiration. In the following sections I will discuss the potential roles for NO inhibition of cytochrome oxidase in a variety of different systems.

3. Smooth and striated muscle

NO first came to prominence when it was found to be identical with the endothelium-derived relaxing factor, responsible for relaxing vascular smooth muscle in response to acetylcholine and other agents in the lumen of blood vessels [5]. NO is produced by endothelial cells in response to these agents, and diffuses to the surrounding smooth muscle, where it causes relaxation and thus dilation of the vessel. The mechanism by which NO causes relaxation of smooth muscle is unclear, although it is generally assumed to act via guanyl cyclase. Could NO inhibition of cytochrome oxidase in the smooth muscle cause the relaxation?

Table 2
Levels of NO produced in various biological systems in response to stimulants

[NO] (μM)	System	Stimulus	Ref.
0.45–5.0	Endothelium in aorta	Bradykinin	[20,21]
0.13–0.85	Smooth muscle in aorta	Bradykinin	[20,21]
0.05–5.0	Endothelium (cultured)	Bradykinin	[20,22,23]
1.0–5.0	Platelets (isolated)	Collagen	[24]
0.02–0.14	Blood (fresh whole)	Collagen	[24]
0.01–0.08	Brain slice	Electrical	[25,26]
1.0–4.0	Brain (in vivo)	Ischaemia	[27]
0.4–1.0	Astrocytes (cultured)	I-γ + LPS	[10]
0.5	Smooth muscle (cultured)	Interleukin-1β	[23]

I-γ + LPS is Interferon-γ and endotoxin.

Endothelial cells produce anything from 0.05 to 5 μM NO in response to vascular relaxing agents, for example bradykinin [20–23]. Electrodes inserted into smooth muscle cells of the aorta record a NO level of 130–850 nM in response to bradykinin [20,21]. Exogenous addition of between 10 nm and 10 μM NO causes smooth muscle relaxation. These levels of NO are sufficient to cause a significant inhibition of cytochrome oxidase, the actual inhibition depending on the tissue oxygen concentration. Such inhibition of cytochrome oxidase would lead to a rise in cellular ADP and P_i, which may inhibit the actinomyosin-ATPase [15], and thus potentially cause muscle relaxation.

Such a mechanism would also provide a simple explanation of how local oxygen concentration controls dilation of blood vessels. Decreased oxygen concentrations cause blood vessels to dilate, and the mechanism of this dilation is unclear [31]. If the vascular tone is set by NO inhibition of cytochrome oxidase in vascular smooth muscle, as suggested above, then a decrease in oxygen concentration would automatically cause dilation, due both to decreased competition of oxygen with NO at cytochrome oxidase, and decreased breakdown of NO by oxygen and oxyhaemoglobin.

NO is thought to regulate relaxation in other smooth muscle types, for example, the stomach and intestine, and possibly lungs, bladder and uterus. Thus the occurrence and role of the NO-inhibition of respiration should be considered in these tissues.

Recently NO synthase has been found in type II fibres of skeletal muscle, and inhibition of NO synthase promotes muscle contraction of isolated muscle bundles [32]. This has led to the suggestion that NO may have some role in promoting relaxation in skeletal muscle. Again NO inhibition of cytochrome oxidase might be involved, since decreasing oxygen concentration is well known to decrease skeletal muscle contraction. However, the levels of NO involved in physiological control of skeletal muscle contraction are not known.

Resting skeletal muscle is the main heat producing organ in many small mammals, and resting muscle heat production or oxygen consumption may be controlled by noradrenaline and other vasoactive agents, via actions on the endothelium [33]. Perfusion of skeletal muscle with NO-producing agents causes an inhibition of oxygen consumption and thus heat production [33]. The respiration of isolated skeletal and heart muscle is inhibited by bradykinin (a stimulant of NO production from the endothelium) and the inhibition of respiration is prevented by inhibitors of NO synthase [11,12]. Inhibitors of NO synthase stimulate oxygen consumption of canine hindlimb [29] and the whole body [30]. Thus it has been proposed that vascular NO production controls tissue respiration [29,30], and the likely site of action of NO is cytochrome oxidase.

4. Brain and synapse

NO has been implicated in mediating long-term potentiation and long-term depression in the central nervous system; that is changes in efficiency of synapses due to usage which may be important in memory formation [4]. Levels of NO measured during induction of long-term depression in cerebellar slices were 20–75 nM [26]. The mechanism by which NO might change synaptic efficiency is unknown. It seems unlikely that the acute effect of NO on cytochrome oxidase could be involved in these changes since the inhibition of respiration in nerve terminals and astrocytes is rapidly reversible [7,10] while the changes in synaptic efficiency are longer term. It seems more likely that the NO inhibition of cytochrome oxidase would cause a acute depression of synaptic activity, since anoxia or respiratory inhibition causes rapid inhibition of action-potential-dependent glutamate release from isolated nerve terminals [19]. Indeed it has been reported that NO mediates an acute synaptic suppression at developing neuromuscular synapses, which was suggested to be involved in synaptic plasticity and possibly muscle fatigue [34]. NO has been reported to mediate growth arrest of cultured PC12 cells differentiating into neurons in response to nerve growth factor [35]; again the inhibition of growth and DNA synthesis might be mediated by an inhibition of ATP production.

Local changes in blood flow and metabolism occur within the brain in response to functional activation, e.g. visual stimulation [2]. The mediator between functional activation of neurons and increased blood flow is now thought to be NO [2]. Interestingly the increased local blood flow is not matched by an increase in oxygen consumption, but rather an activation of glycolysis, thus the local oxygen tension increases. It is conceivable that the increased NO level, resulting from functional activation and causing the increased blood flow, may also locally inhibit cytochrome oxidase and thus be responsible for depressing local oxygen consumption during functional activation.

5. Cytotoxicity

NO release from a whole range of cells is thought to be involved in killing pathogens [1,3,36]. Neutrophils, platelets and endothelial cells, which express the constitutive form of NO synthase, can acutely produce NO in response to activation, which may be involved in killing pathogens. Endotoxins and/or cytokines can induce the expression of the inducible form of NO synthase in macrophages and cells within virtually every tissue in the body. This response has been suggested to act as a kind of primitive immune response, whereby the induced NO production kills pathogens locally. Activated macrophages can kill bacteria, tumour cells, and a variety of other pathogens; and one of the cytotoxic agents is thought to be NO. The mechanism of cytotoxicity of NO is unclear, but is thought to involve damage to: mitochondrial complexes I and II, mitochondrial aconitase, glyceraldehyde-3-phosphate dehydrogenase, ribonucleotide reductase, and DNA [1]. An acute inhibition of mitochondrial cytochrome oxidase or bacterial oxidases

might also contribute to the killing of pathogens. The cytochrome oxidase activity of *Paracoccus denitrificans* has been found to be sensitive to NO, and in some conditions cellular respiration is inhibited by the NO generated by the cells themselves [8].

Expression of the inducible form of NO synthase is part of the signal transduction mechanism by which cytokines and bacterial endotoxins induce inflammatory responses [3,5,36]. Inflammatory responses probably mediated in part by NO include vasodilation, adhesion of leucocytes, microvascular permeation, and killing of pathogens. An excessive or chronic inflammatory response has been implicated as being pathological in a large range of disease processes, including neurodegenerative diseases. High levels of NO will damage or kill many cell type; thus excess NO has been suggested to be involved in pathology of sepsis, endotoxemia, arthritis, asthma, diabetes, multiple sclerosis, Alzheimer's disease, Parkinson's disease, AIDS dementia, and ischaemic damage to brain and heart [1–5,36].

Cultured vascular smooth muscle cells activated by interleukin-1β produce 0.5 μM NO at their surface [23]. Cultured astrocytes activated by endotoxin and interferon-γ have been measured to produce 0.4–1.0 μM NO in dilute incubations [10]. Thus the levels of NO produced in response to cytokines and endotoxin appear sufficient to inhibit cytochrome oxidase. And indeed in the activated astrocytes it was shown that cytochrome oxidase was reversibly inhibited by the NO produced, resulting in a strong inhibition of respiration [10]. These cells also have an irreversible inhibition of cytochrome oxidase and complex I due to long-term exposure to NO [37]. The irreversible damage might be a consequence of reversible inhibition of cytochrome oxidase, since inhibition of cytochrome oxidase is well known to greatly stimulate the production of reactive oxygen intermediates (H_2O_2 and O_2^-) from the mitochondrial respiratory chain, and these metabolites may damage many cellular systems. This might be a general mechanism for converting from reversible inhibition by NO to irreversible damage. It has been proposed that NO directly damages iron–sulphur centres in the mitochondrial electron transport chain [1], but it now appears that these centres are relatively insensitive to direct attack by NO [38].

Since induction of the inducible form of NO synthase by cytokines and/or endotoxin causes production of NO to levels sufficient to substantially inhibit cellular respiration via cytochrome oxidase [10], it seems likely that this inhibition mediates a general metabolic suppression in conditions where NO synthase is known to be induced, such as sepsis, inflammatory disease, neurodegenerative disease, and post-ischaemia (Fig. 1). This might explain, for example, the inhibition of glucose and protein synthesis in cells expressing the inducible form of NO synthase [39], or the inhibition of contractile function in heart following exposure to endotoxin [40].

6. Conclusion

NO almost certainly acts on cells by a variety of different effector systems. There is now growing evidence that one of these effector systems is cytochrome oxidase.

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