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Nitric oxide and hypoxia

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Abstract

NO (nitric oxide) can affect mitochondrial function by interacting with the cytochrome *c* oxidase (complex IV) of the electron transport chain in a manner that is reversible and in competition with oxygen. Concentrations of NO too low to inhibit respiration can trigger cell defence response mechanisms involving reactive oxygen species and various signalling molecules such as nuclear factor κB and AMP kinase. Inhibition of mitochondrial respiration by NO at low oxygen concentrations can cause so-called metabolic hypoxia and divert oxygen towards other oxygen-dependent systems. Such a diversion reactivates prolyl hydroxylases and thus accounts for the prevention by NO of the stabilization of hypoxia-inducible transcription factor. In certain circumstances NO interacts with superoxide radical to form peroxynitrite, which can affect the action of key enzymes, such as mitochondrial complex I, by S-nitrosation. This chapter discusses the physiological and pathophysiological implications of the interactions of NO with the cytochrome *c* oxidase.

Introduction

Following the identification of EDRF (endothelium-derived relaxing factor) as NO (nitric oxide) and the subsequent demonstration of its biochemical synthesis from the semi-essential amino acid L-arginine, the term 'the L-arginine:NO pathway' was coined and its presence was confirmed in many different tissues

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(see [1] for review). This was followed by the elucidation of a number of different biological roles for NO throughout the body. Many of these physiological actions of NO, such as its vasodilator, anti-aggregatory and neurotransmitter properties, result from activation of the soluble guanylate cyclase. In addition, NO can affect mitochondrial function by inhibiting the cytochrome *c* oxidase (complex IV) of the electron transport chain. The physiological and pathological consequences of such inhibition will be discussed in this chapter. We regret that, owing to editorial constraints, we have been unable to cite fully those who have contributed to this field of research.

Synthesis of NO in the cell

Three distinct isoforms of NOS (nitric oxide synthase), with different tissue localization, catalytic properties and regulatory mechanisms, have been identified. These are nNOS (neuronal NOS or NOS-I), eNOS (endothelial NOS or NOS-III) and iNOS (inducible NOS or NOS-II). The genes responsible for the expression of these proteins are known and the X-ray structure of the enzyme isoforms has been determined (see [2] for review). The enzyme catalyses the synthesis of NO from L-arginine and oxygen, as shown in reaction (1):

$$\text{L-Arginine}+2O_2+3/2\text{NADPH}+3/2H^+=\text{Citrulline}+2H_2O+3/2\text{NADP}+\text{NO} \quad (1)$$

In cells, all three isoforms occur as dimers, each monomer containing the following redox centres: FAD, two FMN molecules, iron protoporphyrin IX (haem) and tetrahydrobiopterin. Although the initial nomenclature was based on the tissue in which it was identified, it is now clear that each isomer has a widespread distribution (for review see [2]).

The existence of a mitochondrial isoform of the enzyme (termed mtNOS) has been proposed; however, to date this remains controversial [3]. It is now clear that there is no mtNOS localized to the mitochondrial matrix and that, most likely, a cytoplasmic isoform is associated with the mitochondrial membrane. Studies in endothelial cells have suggested that eNOS is localized at the cytoplasmic face of the outer mitochondrial membrane [4]. Another study has demonstrated a protein–protein interaction between mitochondrial nNOS and the cytochrome *c* oxidase in nervous tissue [5].

Generation of NO close to its site of action remains an intriguing possibility. In this context, the recent observation of NOS inhibitor-independent, nitrite-dependent, generation of NO by isolated yeast and rat liver mitochondria and by isolated cytochrome *c* oxidase at low oxygen concentrations [6] suggests an alternative mechanism whereby mitochondria themselves might generate NO from nitrite, initiating a hypoxia-signalling pathway. Furthermore, nitrite has been suggested to be an endocrine depot of NO, so that the nitrite reductase activity of deoxymyoglobin releases NO close to the mitochondria and thus regulates respiration via cytochrome *c* oxidase [7]. In addition, in the absence of oxygen, eNOS has been reported to catalyse the reduction of nitrite to NO [8]. Whether any of these alternative mechanisms

involved in the generation of NO occur *in vivo* and/or play a role in the regulation of the cytochrome *c* oxidase or in cell signalling remains to be confirmed.

Interaction of NO with the mitochondrial respiratory chain

NO, a gas with a molecular mass and structure close to that of oxygen, has been known for more than 100 years to interact with transition metals and induce spectral changes in methaemoglobin by binding to the haem iron. It is not surprising, therefore, that the interaction of NO with cytochrome *c* oxidase, the terminal enzyme of the mitochondrial respiratory chain, was described long before the biological significance of NO was recognized in the 1980s. The inhibitory effect of NO on cellular cytochrome *c* oxidase was demonstrated in 1994 by several groups (for review see [9]). The inhibition is reversible and in competition with oxygen. The affinity of cytochrome *c* oxidase for NO is greater than that for oxygen, with a K_i of around 0.2 nM for competitive binding [10]. This indicates that interactions between oxygen and NO occur at physiological concentrations of both gases as has been demonstrated in a number of studies during the past few years. In vascular endothelial cells endogenous concentrations of NO modulate cell respiration in an oxygen-dependent manner [11] and in isolated canine cardiac muscle both exogenous and endogenous NO have been shown to reduce the consumption of oxygen [12]. The inhibition of cytochrome *c* oxidase by nanomolar concentrations of NO *in vitro* takes place via two different mechanisms so that NO can block the association of oxygen with the enzyme by binding to: (i) oxidized Cu_B^{2+}, reducing it to give a short lived Cu_B^{+}–NO^{+} intermediate with subsequent formation of nitrite (NO_2^{-}) (k_{on}=10^5 $M^{-1}{\cdot}s^{-1}$); and (ii) the iron atom of the reduced cytochrome a_3 with formation of the a_3^{2+}–NO intermediate (k_{on}=10^8 $M^{-1}{\cdot}s^{-1}$ and k_{off}=0.004 s^{-1} at 20°C). This is light-sensitive, strictly competitive with oxygen and mimicked by carbon monoxide. In both situations the net effect of NO interacting with cytochrome *c* oxidase is the reduction of the enzyme (for review see [10]).

We have demonstrated that inhibition of respiration by NO only occurs after the spare capacity of cytochrome *c* oxidase for oxygen is fully utilized by NO [13]. At lower concentrations NO reduces the electron transport chain without affecting its respiratory efficiency (see below). Since this latter effect of NO is the one associated with cell signalling [14], it is likely that the fine regulation of the behaviour of the enzyme by NO is indeed a physiological mechanism and occurs at concentrations of NO similar to those that activate the soluble guanylate cyclase enzyme, an interaction that is, incidentally, independent of oxygen concentration.

The interaction between NO and cytochrome *c* oxidase *in vivo* remains to be investigated fully; however, it has already been reported that administration of a NOS inhibitor or NO scavenger increases oxygen consumption in

the heart, kidney, skeletal muscle and whole body during rest and exercise in conscious dogs [15], indicating the direct involvement of NO in regulation of mitochondrial respiration.

Free radical formation and NO

In 1971 the 'inhibitor-sensitive' generation of H_2O_2 (hydrogen peroxide) by metabolically efficient mitochondria was investigated and found to represent up to 2% of the total oxygen consumption in intact rat liver mitochondria [16]. Later it was shown that the initial ROS (reactive oxygen species) produced by the respiratory chain is superoxide radical ($O_2^{\bullet -}$) and that this is converted into H_2O_2, either non-enzymatically or by SOD (superoxide dismutase). H_2O_2 is a more stable, membrane-permeable molecule that can diffuse from the mitochondrial matrix to the cytoplasm and out of the cell and has been implicated in cell signalling (see below).

Respiratory complexes I and III are responsible for the one-electron reduction of molecular oxygen resulting in the production of $O_2^{\bullet -}$. At complex III the reaction occurs rapidly only if the physiological intramolecular electron transfer within the enzyme is disrupted by specific inhibitors (e.g. antimycin A). The unstable semiquinone located in the Q_o site is generally accepted to be the direct reductant of molecular oxygen (Figure 1). The production of $O_2^{\bullet -}$ by intact complex III is highly dependent on the mitochondrial membrane potential, as would be expected if it is produced at the Q_o site. Because of the absence of a clear mechanistic model of complex I there is less consensus on the site of generation of $O_2^{\bullet -}$ in this enzyme. However, recent data indicate that reduced flavin or its semiquinone anion is the most likely point of one-electron leak in complex I [17,18]. In contrast with complex III, none of the known redox centres of complex I are located in the

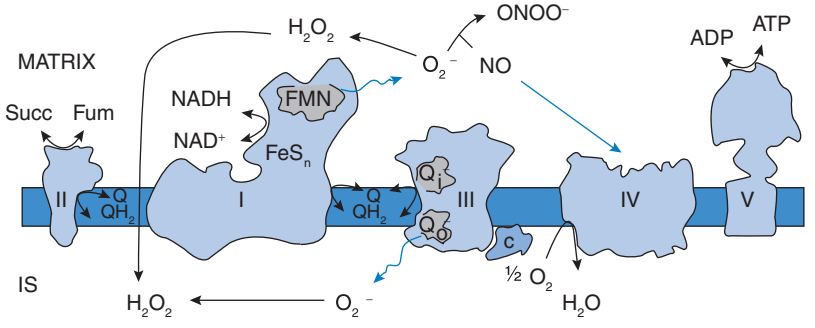

Figure 1. Scheme of the respiratory chain

Inhibition of cytochrome *c* oxidase (complex IV) by NO results in over-reduction of upstream redox centres and enhancement of superoxide radical ($O_2^{\bullet -}$) formation, most likely from complex I. The newly-formed $O_2^{\bullet -}$ is either efficiently dismutated by the mitochondrial Mn-SOD or, in certain circumstances, reacts with available NO to form $ONOO^-$. Succ, succinate; Fum, fumarate; IS, intracellular space.

membrane domain and even during uncoupled respiration all FeS clusters are in a reduced state. Therefore no significant enhancement of complex I-mediated $O_2^{\bullet-}$ production by changes in membrane potential would be expected, as has been shown experimentally [17,18]. The main feature of the generation of ROS by complex I is that the enzyme catalyses $O_2^{\bullet-}$ generation by both direct oxidation of NADH by ubiquinone and a reverse electron transfer reaction from ubiquinol to NAD^+ [18], releasing $O_2^{\bullet-}$ into the mitochondrial matrix. Therefore it is likely that *in situ*, in the absence of inhibitors, complex I rather than complex III is the major contributor to mitochondrial ROS generation during oxidation either of the substrates of NAD^+-dependent dehydrogenases or of succinate.

Reduction of the cytochrome *c* oxidase by NO at low oxygen concentrations has been shown in whole cells to be associated with the release of ROS and subsequent signalling for the activation of NF-κB (nuclear factor κB) [14] and AMP kinase [19]. It has also been demonstrated that increases in NO generation lead to reduction of cytochrome *c* oxidase at oxygen concentrations well above the hypoxic range, thus suggesting that this is not, strictly speaking, a hypoxic ROS-dependent signalling mechanism but that it depends on the relative ratio of NO to oxygen and is a stress response of wider biological significance [14]. Whether the release of ROS is due to the simple backlog of electrons that results from inhibition of the cytochrome *c* oxidase, or from the accumulation of reducing equivalents such as NADH and $FADH_2$ is not clear at present. The NO-induced enhanced reduction of the chain at higher oxygen concentrations leads to a situation that favours the interaction between electrons and oxygen; this is clearly different from the reduction of the chain at the very low oxygen concentrations of hypoxia [19].

At high concentrations exogenous NO has been shown to enhance the generation of H_2O_2 and $O_2^{\bullet-}$ in submitochondrial particles, rat heart mitochondria [20] and Langendorf preparations of isolated heart [21]. Whether this can be explained by the mechanisms above is not clear. In the presence of oxygen high NO concentrations may initiate a series of redox reactions with the consequent release of ROS or products of the reactions between NO and $O_2^{\bullet-}$. Full inhibition of respiration, with complete reduction of the cytochrome *c* oxidase and the entire respiratory chain, may not lead to the generation of ROS, as has indeed been shown using cyanide, which produces full inhibition of respiration without any significant increase in ROS production.

Interaction between NO and $O_2^{\bullet-}$ was identified early on during the quest for the identification of NO itself [1]. Later it was found that this interaction leads to the formation of $ONOO^-$ (peroxynitrite) and occurs at near-diffusion-limited rates ($6.7\times10^9\,M^{-1}\cdot s^{-1}$) that are even faster than the dismutation of $O_2^{\bullet-}$ by SOD. Direct measurements of $ONOO^-$ are not possible *in vivo*; however it has been measured indirectly by its ability to nitrosate proteins and these have been found in a number of pathologies in humans [22]. The source of $O_2^{\bullet-}$ for $ONOO^-$ generation has been widely speculated. Although

the massive emission of highly reactive $O_2^{\bullet -}$ from mitochondria is improbable in the cell under physiological conditions, during pathological conditions the reaction between NO and $O_2^{\bullet -}$, coming from the mitochondria or from other sources (see below), is likely to occur. The formation in mitochondria of $ONOO^-$ from NO can take place only in the vicinity of the enzymes generating $O_2^{\bullet -}$ owing to the short half-life of $O_2^{\bullet -}$ and the high hydrophobicity of NO.

The precise conditions under which $ONOO^-$ is generated are unclear; however, long-term exposure of cells to NO leads to inhibition of cytochrome *c* oxidase that is followed by a gradual and persistent inhibition of complex I, concomitant with a reduction in the intracellular concentration of reduced glutathione [23]. We have suggested that this is likely to be the result of S-nitrosation of critical thiols in complex I since it can be reversed by exposing the cells to high-intensity light or by replenishing intracellular reduced glutathione. Thus, although NO regulates cell respiration physiologically by its action on cytochrome *c* oxidase, long-term exposure to high concentrations of NO leads to persistent inhibition of complex I and potentially to cell pathology. Whether the sequential inhibition of cytochrome *c* oxidase, then complex I, occurs in pathological conditions such as inflammation or degenerative disease is not known.

In spite of the above, there remain many uncertainties in relation to the extent to which mitochondria are a source of ROS under physiological and pathological conditions. Relatively little data have been published on mitochondrial production of H_2O_2 by metabolically competent mitochondria in the absence of inhibitors, and studies on the release of ROS from the mitochondrial respiratory chain in cells in real-time are still in their infancy [13,14]. Furthermore, although the respiratory chain is generally considered to be the main source of mitochondrial $O_2^{\bullet -}$ production, there are other enzymes in the matrix and outer mitochondrial membrane that are able to generate $O_2^{\bullet -}$ and/or H_2O_2. These include: DT-diaphorase, the hydrolipoamide dehydrogenase component of α-ketoglutarate dehydrogenase and pyruvate dehydrogenase complexes, monamine oxidase, dyhydroorotate dehydrogenase and α-glycerophosphate dehydrogenase (see [24] for review). To what extent any of these enzymes contributes to mitochondrial generation of ROS is unknown at present.

There is, in addition, little current data on the relative contribution of different cellular organelles and tissue-specific enzymes to the overall production of ROS in cells. Although the mitochondria and their respiratory chain enzymes, because of their strategic role in oxygen utilization, are likely to be the initiators of any generation of $O_2^{\bullet -}$ and subsequent signalling it is important to consider that even the earliest studies [25] found the mitochondria from rat liver to be responsible for only 15% of the total cellular H_2O_2 production, with the rest being generated by peroxisomes and microsomes. How this translates into the whole cell or the *in vivo* situation remains to be determined. Interestingly, mice lacking the gene encoding Mn-SOD, the

enzyme located in the mitochondrial matrix, exhibit perinatal or early postnatal mortality which does appear to implicate the mitochondria as a major source and target of $O_2^{\bullet -}$ (see [26] for review). It is probable that other non-mitochondrial enzymes contribute to the total production of ROS in cells. So far, the activation of enzymes such as NADPH oxidases, xanthine oxidase and vascular cytochrome P450 has been implicated and substantial evidence now exists showing that the activity as well as the expression of these enzymes can be enhanced by pathological stimuli [27]. Furthermore, NOS has the capacity to generate $O_2^{\bullet -}$ under specific circumstances in which the enzyme becomes uncoupled [28]. The uncoupling of eNOS has been demonstrated in several pathological conditions such as diabetes, hypercholesterolaemia and hypertension. A more subtle mechanism for generation of $O_2^{\bullet -}$ by eNOS has been suggested to occur through the phosphorylation/dephosphorylation of a specific Thr^{495} which might act as a molecular switch between the generation of NO and that of $O_2^{\bullet -}$ [29]. Thus the whole topic of the release of ROS in cells is an evolving field with a great deal of research yet to be carried out. The fundamental questions that remain are to what extent the release of ROS is a physiological mechanism and how is this release modified during cell defence and pathophysiology?

S-nitrosation of proteins

S-nitrosation of thiol groups by the interaction of NO with cysteine has been suggested to be an important post-translational protein modification that plays a role in cell signalling [30]. Although S-nitrosation has been demonstrated in isolated enzymes *in vitro*, in cells and in organs, the mechanism by which it occurs remains unclear. Several possibilities that have been proposed include the reaction of NO itself with a pre-existing $RS^{\bullet}$ (thiyl) radical; with oxygen to form the electrophilic nitrosating agent N_2O_3, or with a transition metal to form the reactive electrophile NO^+. Peroxynitrite has also been suggested to oxidize cysteine residues to form S-nitrosoproteins. Since S-nitrosation appears to be targeted to specific sites on particular proteins some authors have suggested the existence of certain cysteine-containing amino acid consensus motifs in proteins promoting the nitrosating reaction. The pK_A of the cysteine group (from 8 to 10) may contribute to its reactivity, as may the proximity of the target protein to the NO generator (for review see [31]).

Since gaseous NO is a very poor nitrosating agent and has a relatively short half-life in aqueous medium, it is likely that transnitrosation from one thiol group to another could account for some nitrosation reactions. Glutathione, the most abundant thiol-containing peptide in cells, has been suggested to be the potential main depot of the nitrosothiol available for transnitrosation. Some enzymes (thioredoxin reductase, glutathione-dependent formaldehyde dehydrogenase, SOD, glutathione peroxidase) have been shown

to catalyse the reductive cleavage of S-nitrosoglutathione, as does protein disulfide isomerase. Transnitrosation between S-nitrosated proteins and S-nitrosothiols may also facilitate the transfer of NO from extracellular to intracellular thiol pools (for reviews see [30,31]).

During the last decade many proteins have been reported to undergo regulatory modification by S-nitrosation: these include serum albumin, glyceraldehyde-3-phosphate dehydrogenase, creatine kinase, caspase, actin, haemoglobin, ryanodine-sensitive calcium release channels, methionine adenosyl transferase and NMDA receptors (for review see [30]). The list of NO-susceptible proteins, including receptors, enzymes and transcription factors, is extending continually as new examples of regulatory S-nitrosation are added. Since S-nitrosation of a protein is likely to alter its activity, such a modification may have biological relevance. It has been shown, for example, that the NO-dependent inhibition of NOS previously demonstrated [32] may be due to S-nitrosation (see [31]). Also, the anti-apoptotic action of NO has been suggested to be due to S-nitrosation of caspase-3 at Cys^{163}, resulting in enzyme inactivation. This S-nitrosation of the caspase has been shown to occur via transnitrosation by thioredoxin-Cys^{73}-SNO in a reaction that occurs 100 times faster than that of S-nitrosoglutathione and is specific for Cys^{163} of caspase [33].

As discussed above, we found that persistent inhibition of complex I following long-term inhibition of cytochrome *c* oxidase by NO was most probably due to S-nitrosation of this enzyme [23]. This has been confirmed by other research groups [34,35]. Our work [36] and that of others [37] has also indicated that mitochondria are crucial for the formation of S-nitrosoproteins, possibly because of their capacity to generate $O_2^{\bullet -}$ and, as a result, $ONOO^-$. More recently it has been shown that, in addition, S-nitrosation of complex I increases the production of H_2O_2 by mitochondria [38].

It is still not clear whether these processes are linked with physiological signalling responses of the cell or are the beginning of an early response to oxidative stress and pathology. It has been claimed that S-nitrosation of complex I is protective in rat hearts subjected to ischaemia reperfusion injury since the S-nitrosated enzyme can degrade and release NO [34]. Furthermore, altered blood levels of S-nitrosated proteins are associated with impaired clinical outcome in patients with cardiovascular disease [39]. Conversely, inhibition of complex I by S-nitrosation has been implicated in a number of pathologies, including Huntington's, Alzheimer's and Parkinson's diseases [34]. Furthermore, decreased respiratory chain complex I activity has been reported in skeletal muscle biopsies taken from critically ill patients in septic shock [40].

Hypoxia, diversion of oxygen and NO

The intracellular concentration and the dynamics of oxygen redistribution between mitochondria and cytosol are likely to be tissue-specific. Hypoxia, a state of oxygen deficiency which is sufficient to cause an impairment

of function, initiates a sequence of cellular responses. A major signalling pathway is via the HIFs (hypoxia-inducible factors). HIF is a heterodimer consisting of two subunits, HIF-1α and HIF-1β. HIF-1β is constitutively expressed and its concentration is not affected by changes in cellular PO_2 (partial pressure of oxygen). HIF-1α, on the other hand, is subjected to degradation under normal conditions. However, in response to hypoxia, its degradation is inhibited and it dimerizes with HIF-1β, resulting in active HIF-1 (see [41] for review). This leads to binding of HIF-1 to DNA and to up-regulation of the expression of specific genes, including those involved in glucose metabolism, angiogenesis and haematopoiesis.

The HIF-1α subunit is continuously degraded by the ubiquitin–proteasome system after hydroxylation of the amino acids Pro^{564} and/or Pro^{402} (see other chapters in this volume) and subsequent binding of the pVHL (von Hippel-Lindau protein). HIF prolyl hydroxylation is carried out by a family of PHDs (prolyl hydroxylases) whose action is impaired in anoxic or low oxygen conditions, leading to decreased pVHL-ubiquitination and increased HIF-1α stability. Interestingly, a defect in the VHL gene is associated with a variety of cancers, particularly renal cancer, since over-accumulation of HIF results in overproduction of proteins important in this disease such as vascular endothelial growth factor [42].

Following the demonstration that high concentrations of H_2O_2 induce stabilization of HIF-1α, Schumacker and co-workers [43] proposed that HIF-1α is stabilized by ROS generated by mitochondria. Evidence for this comes from several observations, including the fact that loss of cytochrome *c* oxidase in cells [44], or the use of a mitochondrial-targeted antioxidant [45], prevent HIF-1α stabilization at low oxygen concentrations. This remains controversial, however, since a great deal of evidence suggests that during hypoxia there is actually a drop in ROS production [26] and that this initiates the signalling pathway leading to stabilization of HIF-1α. Observations that enzymatically generated $O_2^{\bullet -}$ reduces, and SOD increases, HIF-1α levels also suggest an inhibitory role for ROS [46].

The involvement of a free radical mechanism in the stabilization of HIF-1α is supported by some of our experiments in which it is stabilized by NO at ambient oxygen concentrations in a mitochondrial-independent manner [47]. This occurs using high concentrations of NO, which probably stabilize HIF-1α as a result of $ONOO^-$ generation following the interaction between NO and $O_2^{\bullet -}$. Furthermore in some cancer cells we have demonstrated stabilization of HIF-1α by a free radical mechanism involving NO [48]. These experiments are in agreement with data in the literature showing that S-nitrosation of either the PHDs or the HIF-1α molecule can affect its degradation [49]. Moreover, an interaction of free radicals with iron in the PHDs has been suggested as a possible mechanism for their inactivation [50]. Whether, therefore, at some point in the cellular response to decreasing oxygen concentrations, a mitochondrial-dependent formation of ROS synergizes with a progressive

inactivation of the PHDs to stabilize HIF-1α remains an interesting possibility. Indeed, there is evidence for a strong synergism between ROS- and hypoxia-induced stabilization of HIF-1α [47,48].

The term 'metabolic hypoxia' was introduced by Moncada and Erusalimsky [9] to describe the situation in which oxygen is available for mitochondria but the organelle is unable to use it due to inhibition of the respiratory chain by NO. Metabolic hypoxia is likely to have two phases, the first due to the reversible inhibition of cytochrome *c* oxidase and the second to the more persistent inhibition of complex I and other key enzymes [23,51]. Using a system in which a human cell line is transfected so that it generates finely-controlled amounts of NO we have shown that low concentrations cause a rapid decrease in HIF-1α stabilized by exposure of the cells to 3% oxygen [47]. This effect of NO is dependent on the inhibition of mitochondrial respiration since it is mimicked by other respiratory chain inhibitors (rotenone, myxothiazol and cyanide). In addition, the destabilizing effect on HIF-1α of NO and other inhibitors of mitochondrial respiration in those experiments was independent of ROS since it was neither prevented nor enhanced by antioxidants [52]. The effect of inhibition of respiration on HIF-1α stability is dependent on the reactivation of PHDs since we showed that, upon inhibition of mitochondrial respiration in hypoxia, oxygen is redistributed towards non-respiratory oxygen-dependent targets such as PHD so that they do not register hypoxia [52]. These results have been confirmed in other systems [53]. A clear demonstration of oxygen redistribution is the fact that cells lacking mitochondrial DNA, and therefore unable to utilize oxygen, stabilize HIF-1α at lower concentrations than those required by the parent cell [47]. Thus it is possible that NO-dependent diversion of oxygen may be a mechanism whereby enzymes whose activities are reduced in hypoxia may be reactivated, and during pathology it may be the origin of metabolic changes that occur in inflammation and degenerative diseases.

NO and mitochondrial biogenesis

We have demonstrated that NO activates mitochondrial biogenesis, i.e. mitochondrial proliferation and activation, in various types of cells including brown adipose tissues and HeLa cells [54]. This effect of NO, which has been confirmed by others in rat skeletal muscle [55], is dependent on activation of the guanylate cyclase and is mediated by the induction of PGC-1α (peroxisome proliferator-activated receptor γ coactivator 1α), the main regulator of mitochondrial biogenesis and oxidative phosphorylation. We have also shown that mitochondrial biogenesis is reduced in $eNOS^{-/-}$ mice following either exposure to cold or calorie restriction, thus providing further evidence that a NO/cGMP-dependent pathway plays a significant role in the control of mitochondrial biogenesis and the body energy balance [56].

We have shown more recently that in several animal models of obesity eNOS expression and mitochondrial biogenesis are reduced in a manner that

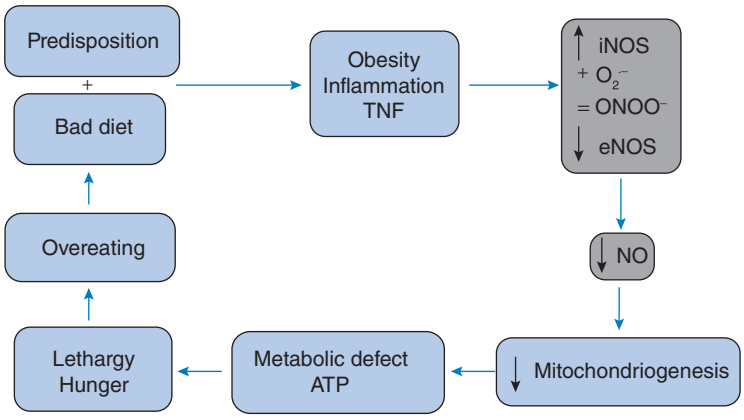

Figure 2. Schematic diagram of the vicious cycle of obesity
It is likely that the steps shown in the grey boxes play a significant role in the pathophysiology of this condition.

can be reversed by preventing the signalling of TNFα (tumour necrosis factor α) [57]. This led us to suggest that reduced mitochondriogenesis in obesity might be the result of the inflammatory reaction, now known to co-exist with adiposity, leading to a switch from eNOS to iNOS activity. We have also suggested that this change may lead to a decrease in NO in favour of NO adducts which will promote further inflammation. Thus it appears as if there is a vicious cycle in obesity in which a genetic predisposition combines with an unhealthy diet to promote obesity and inflammation. Cytokines such as TNFα reduce the availability of NO generated from eNOS, while promoting the generation of iNOS-derived $ONOO^-$, thus reducing mitochondriogenesis. This leads to a lack of ATP production and the resulting hunger and lethargy encourages overeating and greater obesity (Figure 2).

Conclusions

NO plays different roles in multiple biological systems, suggesting a significant regulatory function in cell physiology and also in cell adaptation to stress leading to pathophysiology. These roles are mediated by the well-known activation of the soluble guanylate cyclase, as well as by competing with oxygen at the cytochrome *c* oxidase and most probably via a wide variety of other redox interactions which are at present being actively investigated.

Summary

- *NO interacts with cytochrome c oxidase in a manner that is competitive with oxygen.*
- *This interaction can trigger cell defence response mechanisms involving ROS and various signalling molecules.*
- *In certain circumstances NO interacts with $O_2^{\bullet-}$ to form $ONOO^-$, which can affect the action of key enzymes such as complex I by S-nitrosation.*

- *Inhibition of mitochondrial respiration by NO at low oxygen concentrations can divert oxygen towards other oxygen-dependent systems. Such a diversion reactivates PHDs and thus accounts for the prevention by NO of the stabilization of HIF.*
- *NO promotes mitochondriogenesis by a mechanism involving soluble guanylate cyclase rather than cytochrome c oxidase*

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