Introduction
Inhibition of mitochondrial respiration has been associated with the cytotoxicity of NO right from the beginning of NO research [1,2]. Recent findings have confirmed the importance of mitochondrial inhibition, but put the previous research into a new context. Here we review our findings that: (a) NO reversibly inhibits O2 consumption by isolated cytochrome oxidase, mitochondria, nerve terminals and cells; (b) NO causes glutamate release from brain nerve terminals probably by inhibiting cytochrome oxidase; (c) cultured astrocytes expressing the inducible form of NO synthase (iNOS) reversibly inhibit their own respiration via the inhibition of cytochrome oxidase; (d) mitochondria cause NO breakdown; (e) NO reversibly inhibits catalase; (f) NO and H2O2 react with superoxide dismutase (SOD) to produce peroxynitrite; (g) the reaction between NO and oxyhaemoglobin is much slower in intact blood than in blood after the membranes have been lysed.

NO inhibition of cytochrome oxidase
Cytochrome oxidase is the terminal complex of the mitochondrial respiratory chain, and passes electrons from cytochrome c to O2. Within cytochrome oxidase NO binds to the reduced form of cytochrome a2 and probably also to CuB [3]. This is the same site and form of the enzyme to which O2 binds. It was therefore not surprising that we found that NO inhibits cytochrome oxidase, and this inhibition was apparently competitive with O2 [4]. Others have shown that the NO binding to cytochrome a2 is competitive with O2, but the kinetics and chemistry of the interaction between NO and the O2-binding site of cytochrome oxidase are still unclear [5]. We found that NO rapidly and reversibly inhibited O2 consumption by isolated cytochrome oxidase [4], heart mitochondria [6], brain nerve terminals [4,7] and cultured cells [8]. What was surprising was that the inhibition of cytochrome oxidase occurred at close to physiological levels of NO and O2: O2 consumption of brain nerve terminals (synaptosomes) was half inhibited by 270 mM NO when the O2 concentration was about 150 pM (roughly the arterial level of O2), and half inhibited by 60 nM NO at O2 concentrations of about 30 pM (an average tissue level of O2) [4]. The physiological concentration of NO appears to be in the range 1 nM to 1 μM, depending on tissue and conditions [9]. Thus the NO inhibition of cytochrome oxidase potentially may occur in physiological and/or pathological conditions [9]. Concentrations of NO that inhibited synaptosomal respiration also caused rapid glutamate release from the nerve terminals (measured by adding glutamate dehydrogenase and NADP+, and following the fluorescence of NADPH). This glutamate release was Ca2+-independent, increased at low O2 levels, and was replicated by cyanide (another inhibitor of cytochrome oxidase); it is thus likely to be due to the NO inhibition of cytochrome oxidase. Since glutamate is neurotoxic (particularly in conditions where respiration in the post-synaptic neuron is inhibited), this glutamate release may contribute to NO neurotoxicity.

Reversible inhibition of cellular respiration
It is all very well to show that exogenous NO can cause respiratory inhibition, but can cells producing endogenous NO actually exhibit this inhibition? We tested this by activating cultured cells (astrocytes, the main non-neuronal cell type in the brain) to express iNOS [8]. Many cell types
will express iNOS and produce high levels of NO when activated by inflammatory mediators, such as endotoxin and interferon γ, which we added to the cultured astrocytes for 18 h. We then incubated these cells in a gas-tight vessel with an O₂ electrode and an NO electrode to monitor O₂ and NO levels simultaneously (see Figure 1). The activated cells produced up to 1 μM NO in the medium, and the O₂ consumption of the cells was about half that of control cells. However, this inhibition of respiration was reversible, since inhibition of NOS with a competitive inhibitor (methylarginine) resulted in a stimulation of respiration as the NO was broken down (Figure 1). A subsequent increase in the NO level caused by the addition of arginine, the substrate of NOS, to outcompete the competitive inhibitor resulted in re-inhibition of respiration. Finally, the addition of haemoglobin to completely bind all the NO resulted in full activation of cellular respiration (a 5-fold activation at the low concentrations of O₂ shown in Figure 1). This inhibition of O₂ consumption was attributed to the NO inhibition of cytochrome oxidase based on the NO, O₂ and respiratory substrate dependence of the inhibition [8]. These results indicate that any cell producing high levels of NO will inhibit its own respiration and probably that of surrounding cells.

The NO inhibition of cytochrome oxidase has also been reported by other researchers as (a) an inhibition of respiration in heart submitochondrial particles [10,11], inhibition of respiration in mitochondria isolated from skeletal muscle [12], heart [13] and liver [14], and in synaptosomes [15]; and (b) a reversible depolarization of the mitochondrial membrane potential in isolated liver and brain mitochondria [16], isolated liver cells [17], and isolated β-cells from the pancreas [18].

### Irreversible inhibition of mitochondrial respiration

It is important to distinguish this reversible inhibition of the respiratory chain by low concentrations of NO from previous reports of an irreversible inhibition of the respiratory chain by high concentrations of, or prolonged exposure to, NO [1,2]. However, with physiological or pathological levels of NO (up to 1 μM), we found no irreversible inhibition of respiration in isolated mitochondria [6] or isolated synaptosomes [4,7] with up to 30 min exposure time to these levels.
of NO, and no damage to the mitochondrial EPR-visible iron–sulphur centres [7]. In cultured astrocytes there was no irreversible inhibition of respiration with up to 18 h exposure to endotoxin and interferon γ [8], but after 24 h there was a detectable irreversible inhibition of cytochrome oxidase and other respiratory-chain components [19]. But this irreversible inhibition was probably indirect, and possibly partly due to peroxynitrite [19], as NO may be converted into peroxynitrite by mitochondria-generated superoxide [11]. Peroxynitrite has been shown to irreversibly inhibit mitochondrial respiration at complexes I, II and possibly III, but not complex IV (i.e. not cytochrome oxidase), in contrast with NO [13,20].

Irreversible inhibition of mitochondrial respiration has been associated with iNOS-mediated cytotoxicity in many systems, including macrophages [21,22], hepatocytes [23], myocytes [24], astrocytes [19], vascular smooth muscle [25] and macrophage-mediated killing of tumour cells [26]. Whether this inhibition is due to peroxynitrite, reactive oxygen species (ROS) or other mechanisms is unclear. In some systems this inhibition has been associated with the appearance of a new EPR signal (at g = 2.04) indicative of an iron–sulphur–dinitrosyl species, possibly resulting from damage to mitochondrial iron–sulphur centres. However, when we treated synaptosomes with NO donors we found high levels of this signal without any irreversible inhibition of respiration and no damage to the EPR-visible mitochondrial iron–sulphur centres [7]. Thus the appearance of this signal should not be taken as evidence for damage to mitochondrial iron–sulphur centres.

**NO breakdown by mitochondria and blood**

In addition to NO inhibiting cytochrome oxidase, we have found that isolated mitochondria may cause NO breakdown at a high rate (relative to rates of NO breakdown by intact tissue) [6]. This NO breakdown was partially inhibited by cyanide and other respiratory chain inhibitors, suggesting that part of the breakdown may be mediated by cytochrome oxidase. Others have found evidence for NO metabolism by mitochondria within Chinese hamster ovary cells [27]. The product of NO breakdown may be N₂O (nitrous oxide, laughing gas) as it has been shown that isolated cytochrome oxidase may reduce NO to N₂O [3,28]. Part of the mitochondrial breakdown of NO may also be due to superoxide production by mitochondria, this fraction varying with the O₂ concentration. Mitochondria stimulated to produce high levels of superoxide have been shown to break down added NO [11] and convert it into peroxynitrite [29].

NO reacts with oxyhaemoglobin to produce nitrate and methaemoglobin. This reaction is extremely rapid, the oxyhaemoglobin level in blood is high, and extracellular haemoglobin can deplete intracellular NO levels. Thus it has been argued that blood oxyhaemoglobin will greatly decrease NO levels throughout the body. We measured the rate of NO reaction with oxyhaemoglobin in freshly isolated human blood by adding NO to diluted blood and simultaneously measuring the NO level within an NO electrode and measuring the conversion of oxyhaemoglobin to methaemoglobin with a diode-array spectrophotometer. The rate of NO breakdown and reaction was approx. 2 orders of magnitude slower in intact blood than in blood where the membranes had been disrupted with saponin, or with a similar concentration of free isolated oxyhaemoglobin. This has several important implications. (1) The rate of NO breakdown by blood in vivo may not be as high as previously concluded from its rate of reaction with free oxyhaemoglobin. Note, however, that the rate is still very high. (2) The rate of NO breakdown by blood may be regulated, and pathologies that disrupt the erythrocyte membrane may cause increased NO breakdown in the blood, potentially affecting vasodilation, blood pressure, platelet aggregation, leucocyte adhesion and inflammation.

**Catalase, SOD and ROS**

In a number of systems it has been found that ROS and NO are synergistic in causing cytotoxicity, and ROS and NO are known to interact in a number of ways [30]. NO is known to bind to the haem of catalase, and we have found that NO reversibly inhibits H₂O₂ breakdown by catalase with a Kᵢ of about 0.2 μM NO [31]. Since cultured astrocytes expressing iNOS produce up to 1 μM NO, catalase may be inhibited in these cells, and indeed iNOS expression caused increased H₂O₂ levels in the astrocytes [19].

Recently, we found that H₂O₂ and NO react with SOD to produce a strongly oxidizing species (probably peroxynitrite), which oxidized dihydro-rhodamine to rhodamine. The reaction was
followed by the disappearance of NO (measured with an NO electrode) and the appearance of rhodamine (measured by fluorescence). The reaction occurred with both Cu,Zn-SOD and Mn-SOD, and was inhibited by cyanide, an inhibitor of SOD. Thus this reaction may contribute to the production of peroxynitrite (or other oxidizing species) in conditions where high levels of both NO and H$_2$O$_2$ are present.

Discussion

Short-term exposure to relatively low levels of NO results in a completely reversible inhibition of respiration at cytochrome oxidase. However, prolonged exposure to high levels of NO, or exposure to NO in the presence of ROS, leads to irreversible inhibition of respiration (at complexes I and II particularly). The conversion from the reversible into the irreversible phase of inhibition may be mediated in part by (a) accumulating damage by peroxynitrite, (b) increased ROS production by mitochondria, (c) increased H$_2$O$_2$ levels due to inhibition of catalase, (d) reaction of H$_2$O$_2$ and NO at SOD to produce peroxynitrite, and (e) secondary effects of cytochrome oxidase inhibition, for example glutamate release from nerve terminals.

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