

## REVIEW

## Demystified . . . Nitric oxide

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The discovery of nitric oxide (NO) demonstrated that cells could communicate via the manufacture and local diffusion of an unstable lipid soluble molecule. Since the original demonstration of the vascular relaxant properties of endothelium derived NO, this fascinating molecule has been shown to have multiple, complex roles within many biological systems. This review cannot hope to cover all of the recent advances in NO biology, but seeks to place the discovery of NO in its historical context, and show how far our understanding has come in the past 20 years. The role of NO in mitochondrial respiration, and consequently in oxidative stress, is described in detail because these processes probably underline the importance of NO in the development of disease.

The discovery of nitric oxide (NO) was the greatest achievement of vascular biology in the latter part of the 20th century. The discoverers were awarded the Nobel Prize in Physiology and Medicine. Publications on all aspects of NO run into thousands. Nevertheless, the fact of the matter is that we have not yet been able to harness our knowledge of NO to provide radical improvements in clinical practice.<sup>1</sup> This is partly because the chemistry and biological actions of NO are remarkably complicated for such a simple molecule. The ubiquitous nature and multiple actions of NO make targeting individual organ systems difficult. Having discovered NO, we must next learn to manipulate its metabolism to combat disease. To do this, we must completely understand its role in the living organism.

This review will describe the mixture of deductive reasoning and serendipity that resulted in the discovery of NO. The review will then attempt to explain the evolutionary "why" of NO, and thus account for its ubiquitous nature. With this background, the review will explore exciting new directions in NO research, which could not even be guessed at the time of its original identification. I hope that the information presented here is interesting, entertaining, and above all, demystifying.

**THE DISCOVERY OF NO**

The first question that a layman asks about the discovery of NO is what precipitated the hunt for it in the first place. The answer lies in a classic puzzle of acetylcholine pharmacology. In experiments conducted on the isolated perfused hind-limb of the cat, stimulation of the sympathetic

nerves caused dilatation of the arteries supplying the skeletal muscle (fig 1).<sup>2</sup> This vasodilation was abolished by atropine (an inhibitor of acetylcholine). The effect of atropine implied that acetylcholine released from sympathetic nerve endings diffused to the arterial smooth muscle and caused it to relax. This is the sympathetic cholinergic vasodilator response, which is thought to increase blood flow to the skeletal muscles as part of the "fight or flight" response.<sup>3</sup> In contrast, when arteries were completely removed from the animal and placed in tissue baths (fig 2), acetylcholine generally had no effect or caused the vessel to contract.<sup>4</sup>

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In the late 1970s, Robert Furchgott began to examine this dichotomy in acetylcholine behaviour. This led to the first serendipitous discovery in the NO story.<sup>5</sup> At this time, isolated arteries were cleared of both adventitia and endothelium to obtain a "pure" smooth muscle preparation. In this circumstance, acetylcholine usually causes a contraction. On one occasion, Furchgott's technician, Zawadzki, did not remove the endothelium in a rabbit aorta preparation, and acetylcholine caused a potent relaxation.<sup>6</sup> Furchgott quickly established that arterial relaxation in response to acetylcholine only occurred if the endothelium was present (fig 3)—that is, vascular relaxation to acetylcholine was endothelium dependent. The relaxation was blocked by atropine, implying that acetylcholine was acting on endothelial cell receptors to produce a substance that could diffuse to the smooth muscle and initiate relaxation: there was an endothelium dependent relaxing factor (EDRF). The difference between *in vivo* and *in vitro* responses to acetylcholine resulted from the fact that *in vivo* preparations retained their endothelium, whereas *in vitro* preparations did not.<sup>3</sup>

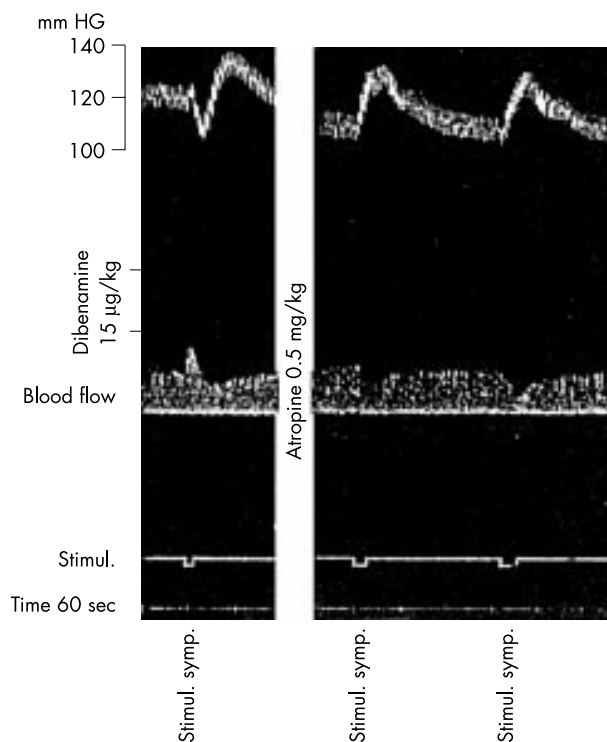
**Elucidating the nature of EDRF: the efficacy of deductive reasoning**

Despite the fact that EDRF was apparently discovered by chance, Furchgott was aware, as were others, that the endothelium is not a passive cellular layer. The release of prostaglandins and

**Abbreviations:** EDRF, endothelium dependent relaxing factor; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; ROS, reactive oxygen species

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Accepted for publication  
4 July 2002

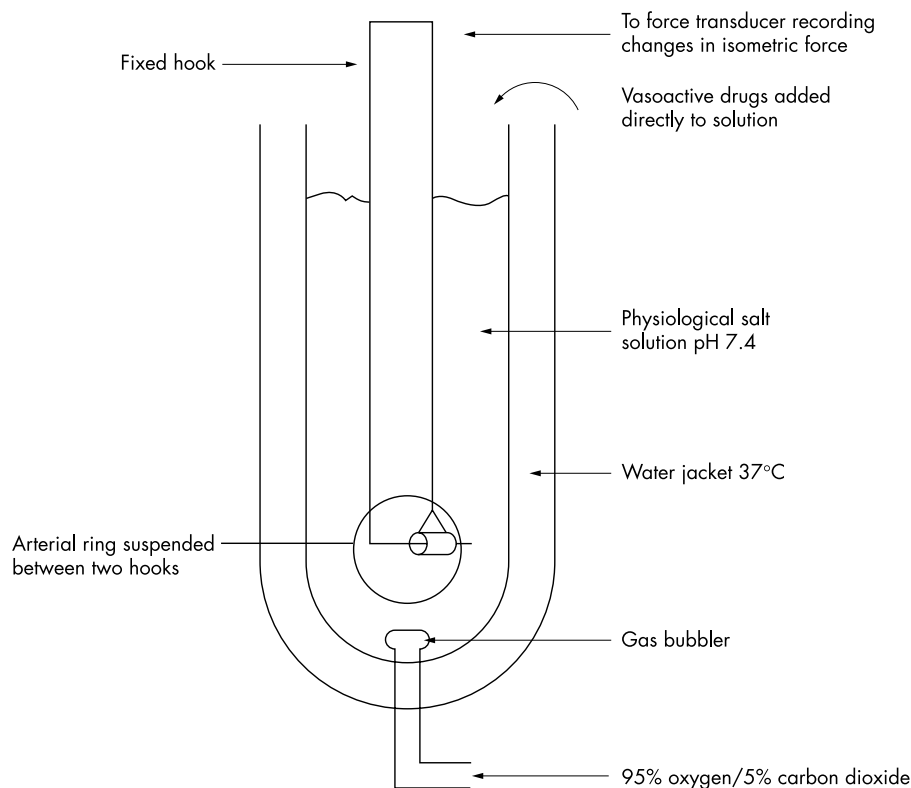


**Figure 1** In this classic experiment by Folkow (1948), the hind limb of an anaesthetised cat was isolated surgically, and blood flow through the limb was measured in response to stimulation of the sympathetic nerves from L3 to L5. This smoke drum trace records changes in limb arterial pressure and blood flow in response to sympathetic stimulation. Under control conditions sympathetic nerve stimulation causes a fall in blood pressure and an increase in flow—that is, vasodilatation (left panel). In the presence of atropine, a muscarinic receptor antagonist, the fall in blood pressure is abolished, and blood flow decreases, indicating a degree of vasoconstriction (right panel) (reproduced with the kind permission of Blackwell Publishing).<sup>2</sup>

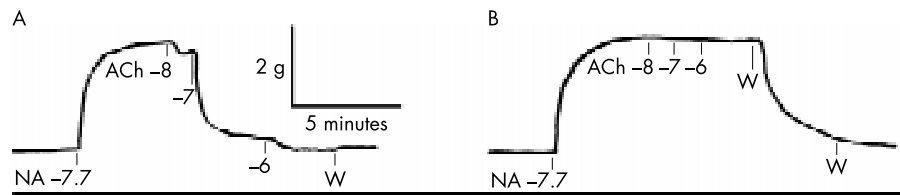
kinins from endothelial cells was already documented.<sup>7-9</sup> The potential interaction between endothelial cell products and platelets was also well known.<sup>10</sup> Thus, the most obvious choice for an endothelium derived relaxing factor would be a vasodilating prostaglandin such as prostacyclin ( $\text{PGI}_2$ ). The vascular endothelium could certainly be induced to manufacture prostacyclin, which then diffused to the smooth muscle to cause relaxation. Nevertheless, inhibition of prostacyclin production did not alter the relaxation to EDRF.<sup>5 11</sup> Thus, although prostacyclin can be an endothelium derived vascular relaxant, it is not EDRF.<sup>12</sup>

Although the identity of EDRF was unknown, many experiments were performed in the early 1980s that elucidated its chemical properties and mode of action. Many workers were actively exploring the role of cyclic nucleotides in vascular smooth muscle relaxation at this time. Prostacyclin was known to cause arterial relaxation by stimulating adenylate cyclase activity in the smooth muscle and so causing a rise in cAMP,<sup>13</sup> but as we have seen, prostacyclin is not the EDRF. By contrast, nitrovasodilators such as glyceryl trinitrate and sodium nitroprusside were shown to initiate vascular smooth muscle relaxation by stimulation of guanylyl cyclase and a rise in cGMP.<sup>14-16</sup> Nitric oxide, which was known to be liberated spontaneously from sodium nitroprusside and glyceryl trinitrate<sup>17</sup> was also shown to increase cGMP,<sup>18</sup> although a direct link with the phenomenon of EDRF was not made at this time.<sup>16</sup> The relaxation induced by nitrovasodilators was inhibited by methylene blue, a vital dye that inhibits guanylyl cyclase, and so inhibits cGMP formation. The relaxation in response to NO was also inhibited by methaemoglobin.<sup>18</sup> Haemoglobin was assumed to act by absorbing the NO, which was known to be an unstable molecule with a short half life.<sup>16</sup>

It was quickly shown that EDRF also caused vascular relaxation through the activation of guanylyl cyclase and thus cGMP (fig 4).<sup>19-21</sup> Furthermore, EDRF was an unstable substance, the action of which was blocked by haemoglobin (fig 4).<sup>20</sup> The short half life of EDRF was determined using an ingenious bioassay technique, which is explained in fig 5.<sup>22</sup> The



**Figure 2** Outline of the classic "organ" or "tissue" chamber set up on which much of the important work in 20th century pharmacology was carried out, including the elucidation of the identity of nitric oxide.



**Figure 3** The famous experiment that first described the existence of an endothelium derived relaxing factor. In these experiments, rings of rabbit aorta were suspended in organ chambers and made to contract with noradrenaline (NA). When the contraction was stable, acetylcholine (ACh) was added cumulatively ( $1 \times 10^{-8}$  M,  $1 \times 10^{-7}$  M, etc). (A) In “unrubbed” rings—that is, rings of aorta in which the endothelium was intact, ACh caused a concentration dependent relaxation. (B) In aortic rings where the endothelium had been deliberately removed by rubbing the vessel lumen, ACh had no effect (or sometimes caused a contraction) (reproduced with the kind permission of the authors and the Nature Publishing Group).<sup>5</sup>

half life of EDRF could be considerably prolonged by the addition of superoxide dismutase, a biological scavenger of superoxide ions (fig 5),<sup>22</sup> indicating that EDRF was vulnerable to inactivation by oxygen derived free radicals.

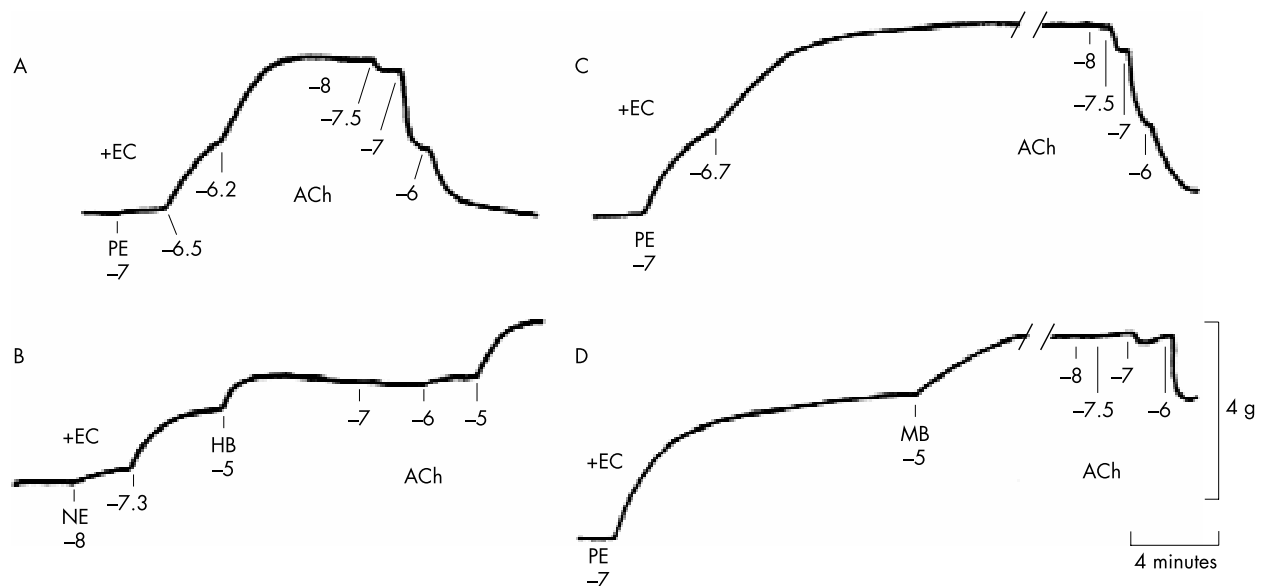
“Nitric oxide is now known to be a ubiquitous signalling molecule, and multiple forms of nitric oxide synthase have been described, specific to particular organ systems and even to individual species”

The similarities between the properties of NO and the properties of EDRF were well recognised by 1986 (fig 6).<sup>23</sup> In 1987, two separate laboratories published definitive evidence that NO was EDRF.<sup>24, 25</sup> The group of Ignarro showed that EDRF derived from the pulmonary artery by bioassay had identical vasodilating properties to NO applied directly to the vascular smooth muscle. Moncada’s group showed that endothelial cells in culture released an unstable vasorelaxant molecule in response to acetylcholine with identical biological activity to EDRF. Using a chemiluminescence technique this laboratory was able to show that the vasoactive molecule was NO. EDRF and NO were conclusively shown to be the same molecule. Shortly thereafter, Moncada’s group showed that NO is derived from the amino acid L-arginine,<sup>26</sup> and that NO synthesis takes place in endothelial cells.<sup>27</sup> NO synthase

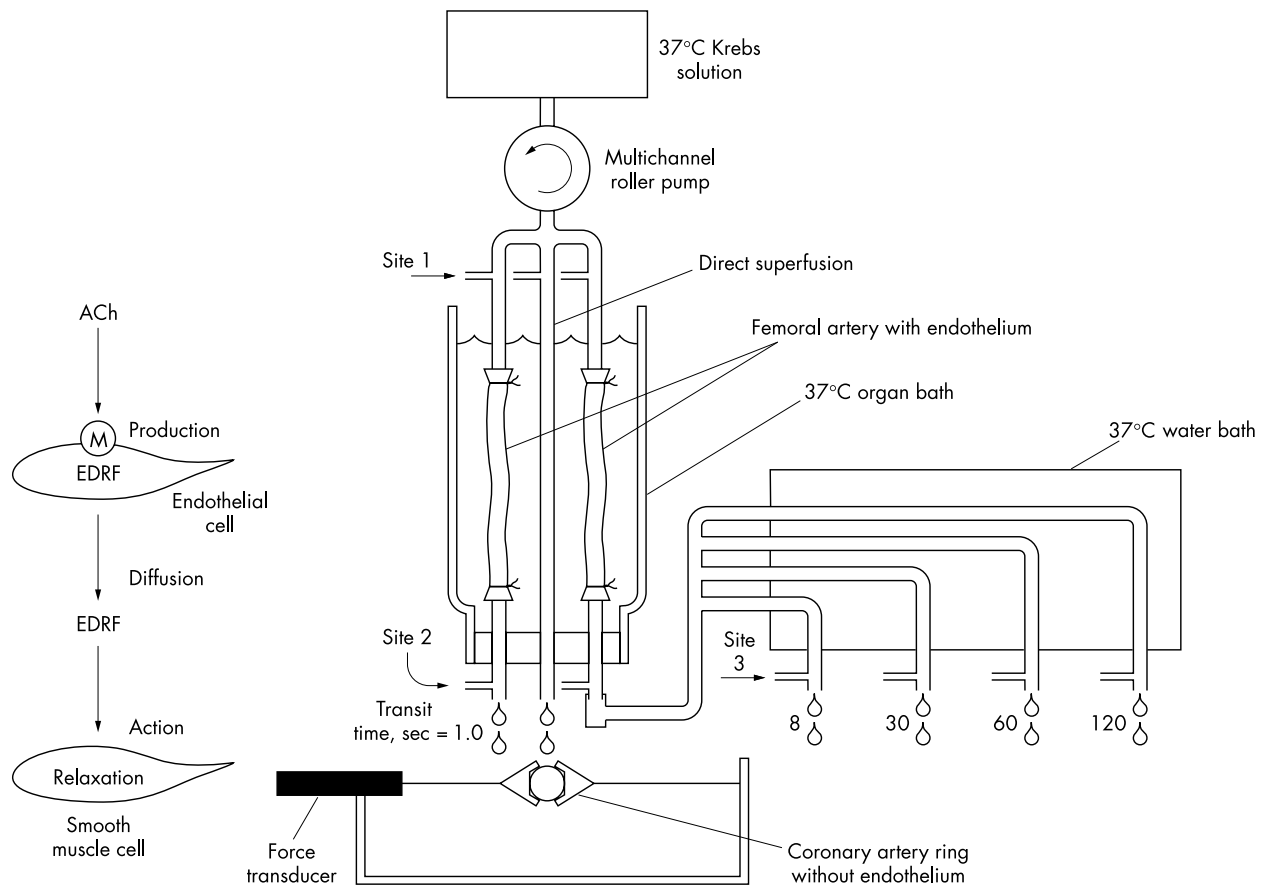
(NOS), the enzyme responsible for the conversion of L-arginine to L-citrulline, with the consequent production of NO, was isolated and purified by Bredt and Snyder in 1990.<sup>28</sup> The time span from the original description of an EDRF phenomenon to the identification of NO and the isolation of the synthesising enzyme was exactly one decade.

### THE UBIQUITOUS NATURE AND EVOLUTIONARY ORIGIN OF NO

NO was originally shown to be a means whereby the endothelial lining of blood vessels communicated with the underlying vascular smooth muscle. NO is now known to be a ubiquitous signalling molecule, and multiple forms of NOS have been described, specific to particular organ systems and even to individual species. Endothelial NOS (eNOS) catalyses the sustained release of small amounts of NO from endothelial cells at rest.<sup>29</sup> eNOS is upregulated by the stimulation of endothelial cell surface receptors (for example, by acetylcholine) or by physical phenomena, such as shear stress.<sup>29</sup> In the presence of bacterial endotoxin, the enzyme inducible NOS (iNOS) is upregulated in macrophages, vascular smooth muscle cells, and endothelial cells.<sup>30, 31</sup> The resulting cascade of NO production is thought to account for the systemic hypotension of septic shock.<sup>32</sup> Neuronal NOS (nNOS) is present in both the central and peripheral nervous systems.<sup>33</sup> In the brain, NO may



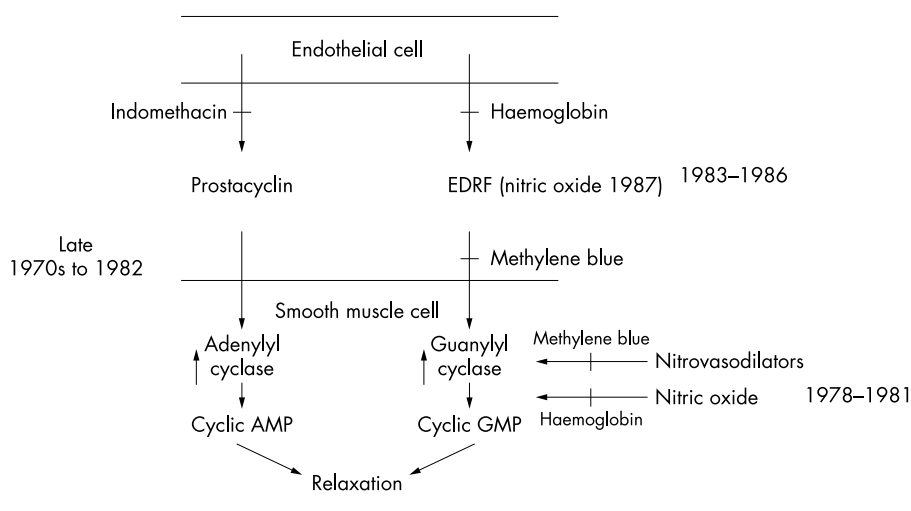
**Figure 4** Similar experiments to those depicted in fig 3 were also carried out using rabbit aorta. In all experiments, a stable contraction is reached with phenylephrine (PE) before the relaxation in response to acetylcholine (ACh) is examined. (A) Vessel ring with endothelium (+EC) relaxes in response to ACh. (B) This vessel ring also has an intact endothelium but the relaxation to ACh is abolished by the presence of haemoglobin (HB). (C) Control response in vessel with endothelium. (D) This vessel has an intact endothelium, but in the presence of the inhibitor of guanylyl cyclase, methylene blue (MB), the relaxation to ACh is abolished and converted into a contraction (reproduced with the kind permission of the authors and the American Society of Pharmacology and Experimental Therapeutics).<sup>20</sup>



**Figure 5** This rather complex set up helped to establish the diffusibility and the transient nature of endothelium dependent relaxing factor (EDRF). When the ring of canine coronary artery was sitting under the metal pipe (direct superfusion), acetylcholine (ACh) added to the superfusing mixture caused the coronary artery to contract because it had no endothelium. When the superfusing mixture passed through a femoral artery with endothelium, the ACh stimulated the release of an EDRF, which then caused relaxation of the underlying coronary artery. This part of the experiment established that released EDRF could travel a short distance, and thus could diffuse from the endothelial cell to the smooth muscle. To show the transient nature of EDRF, the superfusate from the femoral artery was passed through a series of pipes to increase the transit time before it reached the coronary artery (see right of figure; times are 8, 30, 60, and 120 seconds). The longer the transit time, the smaller the relaxation. The effect of increasing transit time could be partially offset by infusing superoxide dismutase at site 2 or site 3. This experiment indicated that EDRF was an unstable molecule that could be rendered inactive by superoxide ion (reproduced with the kind permission of the authors and the American Physiological Society).<sup>22</sup>

mediate neuronal plasticity, thus initiating the processes of learning and memory.<sup>33</sup> NO accounts for a proportion of non-adrenergic, non-cholinergic autonomic activity, and nNOS is colocalised with both neuropeptides and acetylcholine in the parasympathetic nervous system.<sup>34-36</sup> In the gut, these nerves

mediate the relaxation of the oesophageal and pyloric sphincters, and are important regulators of urogenital function.<sup>37, 38</sup> Because NO is a highly diffusible gas, it cannot be stored in nerve endings and is synthesised de novo on activation of the synaptic ending or astrocyte.<sup>38</sup> Therefore, nNOS is a more



**Figure 6** The sequence of deductive reasoning that led to the discovery of nitric oxide. EDRF, endothelium dependent relaxing factor.

highly regulated form of the enzyme, and efficient NO transmission is highly dependent on the presence of abundant L-arginine.<sup>33–39</sup> nNOS is not confined to the nervous system. The macula densa of the kidney is rich in nNOS, and here NO appears to stimulate renin release,<sup>40</sup> possibly via the induction of cyclooxygenase and a resultant rise in macula densa prostaglandin values.<sup>41</sup> Finally, nNOS is located on the synaptic endplate, sarcoplasmic reticulum, and mitochondria in skeletal muscle, implying multiple actions in skeletal muscle contraction (see Grozdanovic for a review).<sup>42</sup> Dysfunction of skeletal muscle nNOS may account for some forms of muscular dystrophy, although the mechanism of this dysfunction is highly complex.<sup>43</sup>

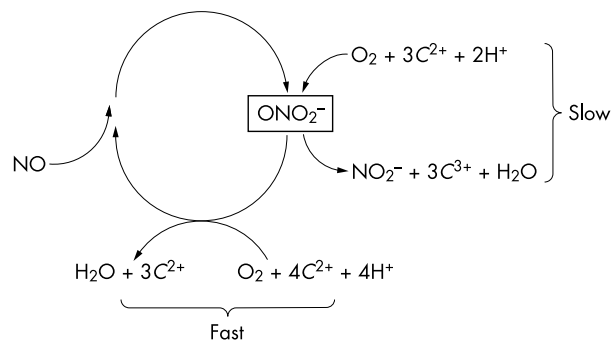
Originally described in mammalian systems, NO is now known to be a ubiquitous signalling molecule across species. An iNOS specific to fish has been described.<sup>44</sup> NO is also a signalling molecule in insects,<sup>45</sup> marine sponges,<sup>46</sup> myxomycetes,<sup>47</sup> and bacteria.<sup>48</sup> In plants, NO induces leaf expansion and root growth<sup>49</sup> and protects against environmental and infection related stresses in a manner similar to human macrophages<sup>50–51</sup> (see section on oxidative stress below).

“Excessive production of reactive oxygen species is implicated in the pathogenesis of several chronic diseases, notably the neurodegenerative disorders Parkinson’s disease and Alzheimer’s disease, and the endothelial destruction and plaque formation typical of atherosclerosis”

These observations suggest that NO is a signalling molecule with a very ancient history, serving biological functions in the most primitive organisms. But why should such a simple gas be so important? Early life obtained nitrogen for the formation of amino acids directly from the atmosphere. Lightning catalysed the conversion of the stable nitrogen molecule (N<sub>2</sub>) to the unstable, and therefore reactive, molecule, nitric oxide (NO•),<sup>52</sup> which could then take part in biochemical reactions. Changing environmental conditions eventually drove the evolution of biological (that is, enzymatic) methods of nitrogen fixation.<sup>52</sup> Nevertheless, it is likely that NO retained its biological role as a signalling molecule and in defence against oxidative stress. By chance, one of the earliest observations made in EDRF (NO) chemistry, the apparent “inhibition” of EDRF by haemoglobin,<sup>20</sup> actually describes an ancient interaction between animal haemoglobins and NO, which enhances oxygen delivery in areas of low O<sub>2</sub> tension.<sup>49</sup>

## NO, OXIDATIVE STRESS, AND SEPSIS

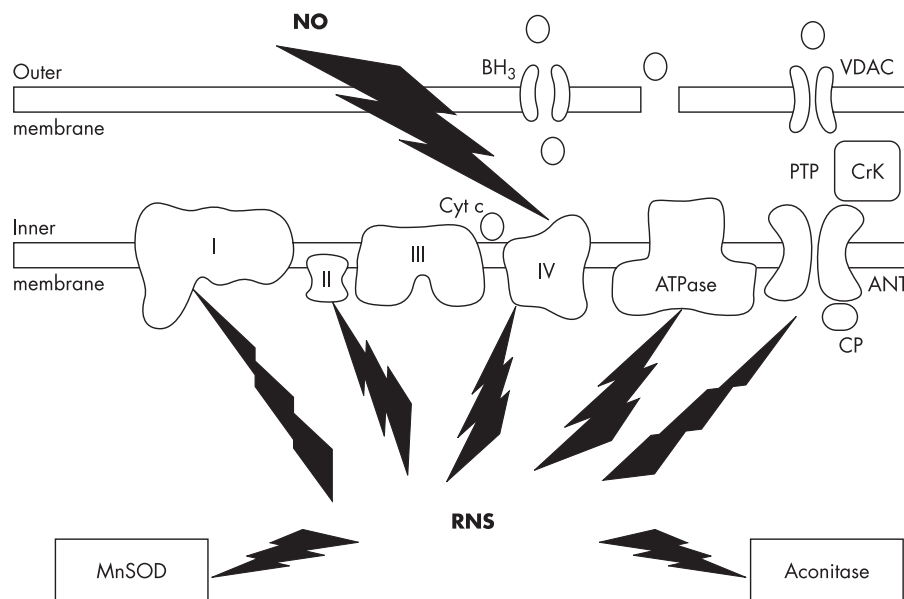
Reactive oxygen species (ROS) such as superoxide radicals, hydroxyl radicals, and hydrogen peroxide form part of the normal signalling and homeostasis mechanisms of all living organisms. They arise from the normal biological activity of cell oxidases (for example, NAD(P)H oxidase, xanthine oxidase) and mitochondrial respiration. Similar to NO (itself a ROS), these unstable and reactive molecules are key mediators in a diverse range of biological activities, such as apoptosis, intracellular signalling, and oxygen sensing (for a review see Droge).<sup>53</sup> Indeed, interest in the physiological roles of these molecules, as opposed to their detrimental effects, was stimulated by the discovery of NO. In contrast, oxidative stress, and thus cellular damage, is caused by the excessive production of ROS. ROS cause cellular damage by interfering with cell respiration, intracellular second messenger systems, and protein synthesis.<sup>54</sup> In addition to the important role of ROS in sepsis,<sup>55</sup> excessive production of ROS is implicated in the pathogenesis of several chronic diseases, notably the neurodegenerative disorders Parkinson’s disease<sup>56</sup> and Alzheimer’s disease,<sup>57</sup> and the endothelial destruction and plaque formation typical of atherosclerosis.<sup>58–59</sup>



**Figure 7** Working hypothesis by Pearce and colleagues for the role of nitric oxide (NO) in mitochondrial respiration. Under normal conditions, protons (4H<sup>+</sup>) and electrons from the citric acid cycle are passed along the electron transport chain, from coenzyme Q to cytochromes b, c<sub>1</sub>, c, and a + a<sub>3</sub> (4c<sup>2+</sup>), generating energy in the process (oxidative phosphorylation). At the end of the chain the electrons and protons combine with oxygen to form water. Cytochrome c oxidase catalyses the final transfer of electrons from cytochrome c to cytochromes a + a<sub>3</sub> (the fast reaction shown at the bottom of the diagram). NO has a high affinity for cytochrome c oxidase, and will bind to this mitochondrial enzyme more readily than does molecular oxygen (O<sub>2</sub>). However, molecular oxygen can diffuse into the active site where the NO is bound, and may gain an electron from one of the metal ion cofactors present (the authors suggest CuB). This results in the formation of superoxide—that is, O<sub>2</sub><sup>-</sup>. The superoxide immediately reacts with the NO, forming peroxynitrite, which then further oxidises the metal ion cofactor to produce water and nitrite ion. Because all intermediates are enzyme associated, no superoxide or peroxynitrite is released into free solution. Thus, under normal physiological conditions, cytochrome c oxidase suppresses peroxynitrite formation by scavenging available NO and preventing it from reacting with superoxide in free solution (the slow reaction shown on the right of the diagram). Because the overall reaction is slow, and the affinity of NO for cytochrome oxidase is high, normal mitochondrial respiration is inhibited. Although this interpretation of the mitochondrial/NO interaction is intriguing, other proposed inhibitory reaction cycles (for example, mechanisms described in: Torres and colleagues<sup>54</sup> and Guiffre and colleagues<sup>65</sup>) are at least equally plausible. Indeed, there may be more than one mechanism operative, depending on whether NO or O<sub>2</sub> is the first to enter the active site (reproduced with the kind permission of the authors and the American Society for Chemistry and Molecular Biology).<sup>63</sup>

“It has been suggested that in times of cell stress, nitric oxide inhibits mitochondrial (aerobic) metabolism, thus reducing oxygen consumption and preventing the onset of apoptosis”

To demonstrate the way in which ROS induce oxidative stress, and the way in which NO can alter these actions, this review focuses on mitochondrial generation of superoxide anion; effectively an oxygen molecule with an additional reactive electron (O<sub>2</sub><sup>-</sup>). Mitochondrial respiration is inherently wasteful: 1–3% of the oxygen entering the electron transport chain is only partly reduced and is thrown off from the cytochrome chain as superoxide.<sup>60–61</sup> There is a complex and poorly understood interaction between mitochondrion derived ROS and NO<sup>62–63</sup> (see full explanation in fig 7). NO has been shown to have an important role as a regulator of mitochondrial respiration.<sup>66</sup> It binds to the mitochondrial respiratory enzyme cytochrome oxidase more readily than does oxygen itself, and thus seems to control the rate of mitochondrial energy production by regulating the rate at which molecular oxygen enters the respiratory chain.<sup>62–66</sup> Because the affinity of NO for cytochrome oxidase is much higher than the affinity of oxygen for the enzyme, mitochondrial respiration could, in theory, be stopped completely by only moderately raised concentrations of NO. It has been suggested that in times of cell stress, NO inhibits mitochondrial (aerobic) metabolism, thus reducing



**Figure 8** Main actions of nitric oxide (NO) and reactive nitrogen species (RNS) on mitochondria. NO specifically and reversibly inhibits cytochrome oxidase (complex IV); RNS inactivate multiple respiratory complexes (I, II, IV), the ATP synthetase (ATPase), aconitase, and Mn superoxide dismutase (MnSOD). RNS activate the permeability transition pore (PTP). Activation of PTP may lead to cytochrome c release either as a result of swelling induced rupture of the outer membrane, or through specific pores. Cytochrome c release may also be induced by the insertion of proapoptotic BH<sub>3</sub> proteins into the outer membrane (reproduced with the kind permission of the authors and Taylor and Francis, Inc, <http://www.routledge-ny.com>).<sup>62</sup> Ant, ATP/ADP translocator; CP, cyclophilin D; CrK, creatinine phosphokinase; VDAC, voltage dependent anion channel.

oxygen consumption and preventing the onset of apoptosis.<sup>67</sup> This cell protective effect of NO has been postulated to offer neuroprotection in the presence of early central nervous system stress.<sup>68</sup>

Mitochondrial cytochrome oxidase may have a second cell protective function: the removal of the toxic derivative of NO, peroxynitrite.<sup>63</sup> NO has a high affinity for ROS, resulting in the formation of highly toxic reactive nitrogen species, including peroxynitrite. At low (physiological) concentrations of NO, these reactions do not generally proceed because the NO is bound to guanylyl cyclase, thus stimulating the formation of cGMP.<sup>69</sup> In pathological conditions such as sepsis, where production of both NO and ROS is massively increased, reactive nitrogen species accumulate rapidly inside the cell (fig 8).<sup>62</sup> These species bind irreversibly to multiple components of the mitochondrial respiratory chain, effectively terminating cell respiration and precipitating cell necrosis.<sup>62</sup> It can be speculated that in severe sepsis, the protective capacity of cytochrome c oxidase is overwhelmed and irretrievable mitochondrial damage occurs. For these reasons, the organ failure characteristic of sepsis is now regarded as a mitochondrial disease, requiring the development of radically different treatment strategies that target mitochondrial enzymes.<sup>70</sup> Mitochondrial dysfunction in the face of excessive ROS formation may also form the basis of many other diseases, and the manipulation of mitochondrial respiratory enzymes may represent the future in the management of oxidative stress (for a review see Lopez and Melor).<sup>71</sup>

### CONCLUSION: THE FUTURE FOR NO

NO was originally described as an endothelium derived relaxant of vascular smooth muscle, but in the past decade it has been shown to have a far more complex and diverse role. The great evolutionary age of the NO molecule probably explains its ubiquitous nature as an intercellular and intracellular messenger. Most important is the very recent discovery of its role as a primary modulator of the mitochondrion, itself a very ancient biological entity. The study of this complex interaction may finally lead to a highly sophisticated understanding of disease and its management.

### Take home messages

- Nitric oxide was first described in the 1980s as an endothelium derived relaxant of vascular smooth muscle, but the past decade has shown that NO is an important signalling molecule in many biological systems
- Because the chemistry and biological actions of NO are remarkably complicated, and because of its ubiquitous nature and multiple actions, we still have much to learn about its role in the living organism
- NO has an important role as a regulator of mitochondrial respiration—it binds to cytochrome oxidase with greater affinity than oxygen itself and may be involved in regulating cellular respiration in times of stress
- Unravelling the role of NO as a modulator of respiration may enable us to manipulate its metabolism to combat disease

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*Mol Path* 2002 55: 360-366

doi: 10.1136/mp.55.6.360

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