Pathological implications of nitric oxide, superoxide and peroxynitrite formation
Joseph S. Beckman and John P. Crow
Department of Anesthesiology, The University of Alabama at Birmingham, Birmingham, Alabama 35233, U.S.A.

Introduction
Approximately 6 years ago, nitric oxide was identified as the endothelium-derived relaxing factor (EDRF), the endogenous vasodilator mimicked by nitroglycerin and nitroprusside [1-4]. An astonishing variety of tissues synthesize nitric oxide, which has important roles in the control of systemic blood pressure, respiration, digestion, penile erection, platelet aggregation, cerebral blood flow and neuronal synaptic plasticity [5]. Furthermore, nitric oxide, or a secondary oxidant derived from it, contributes to the microbicidal and tumoricidal activities of activated macrophages and neutrophils [6, 7]. Endothelium and neurons produce nitric oxide by a calmodulin-activated enzyme, which oxidizes arginine to citrulline and requires biopterin, NADPH and oxygen [8, 9]. The biopterin remains tightly bound to the enzyme and may be recycled within it. Nitric oxide synthase also contains FAD, FMN and ferric haem as essential cofactors. The macrophage enzyme is not regulated by calmodulin, and gene sequences reveal significant differences between the brain, endothelial and macrophage enzymes [10].

Pathological conditions can substantially up-regulate the production of nitric oxide. Stimulated macrophages and neutrophils produce significantly greater fluxes of nitric oxide than endothelium by the inducible calcium-independent nitric oxide synthase. Endotoxin (lipopolysaccharide) and cytokines induce the calcium-independent nitric oxide synthase in many tissues that do not normally produce nitric oxide [11]. For example, the acute hypotension associated with septic shock may be mediated by the expression of the inducible nitric oxide synthase [12].

If nitric oxide is a major cytotoxic agent, then why is nitric oxide generated for signal transduction not toxic? Although the amounts of nitric oxide produced for signal transduction are significantly lower than those produced by macrophages the continuous daily formation of nitric oxide in brain and endothelium will result in vastly greater exposure to nitric oxide (measured as time x concentration) than the transient activation of inflammatory cells for a few hours. In part, the answer is that the toxicity of nitric oxide at low concentrations has been over-estimated due to extrapolation from the rapid formation of other more reactive nitrogen oxides at high concentrations. In millimolar concentrations, nitric oxide rapidly reacts with oxygen to form nitrogen dioxide (NO₂) as well as the strong two-electron oxidants, N₂O₃ and N₂O₄, by dimerization of nitric oxide and nitrogen dioxide. However, the rate of formation of NO₂ is determined by the third order reaction:

\[ k_3 [\text{NO}]^2 [\text{O}_2] \] (1)

Consequently, the reactivity of nitric oxide falls rapidly with the square of nitric oxide concentration. At low concentrations, similar to those which can be produced in vivo, nitric oxide can be administered safely in the gas phase for weeks to treat pulmonary hypertension [13]. While the orange-brown colour of concentrated nitrogen dioxide is formed immediately when gaseous nitric oxide contacts air, this reaction is at least 10 million times slower at the maximum physiological nitric oxide concentrations (about 0.1 µM) [14, 15]. In a simple in vitro system, over an hour is required for physiological concen-
trations of nitric oxide to decompose to nitric and nitrate [16].

Nitric oxide and superoxide

The concentration of nitric oxide in the micro-

 sourrounding area is high enough to produce nitrogen dioxide and thus greatly increase the toxic effects of nitric oxide. However, nitric oxide contains an unpaired electron and readily participates in many free radical pro-

 cesses of pathological relevance. For example, macrophages can also produce superoxide (O\textsubscript{2}\textsuperscript{−}), which reacts with nitric oxide to form the powerful oxidant, peroxynitrite anion (ONOO\textsuperscript{−}).

Primary evidence for establishing the physiological action of nitric oxide or EDRF is that it is destroyed by superoxide and stabilized by superoxide dismutase. Superoxide reacts with nitric oxide at a rate of 6.7(±0.9)×10\textsuperscript{9} M\textsuperscript{−1} s\textsuperscript{−1} to form peroxynitrite [17, 18]. Initially, nitric oxide was viewed as a protective factor because it rapidly scavenges superoxide [19]. However, the reaction product of superoxide and nitric oxide is peroxynitrite (ONOO\textsuperscript{−}), which is a relatively long-lived strong oxidant:

\[
O_2^{\bullet+} + \cdot NO \rightarrow ONOO^{−} \tag{2}
\]

Thus, nitric oxide may substantially increase the toxicity of superoxide by converting a relatively mild reductant into a potent and relatively long-

 lived oxidant [20].

The rate of peroxynitrite formation is determined by the product of concentrations of nitric oxide and superoxide, which are quite low under normal circumstances. Because many pathological processes can simultaneously stimulate the production of nitric oxide and superoxide, the rate of peroxynitrite formation can become substantial under pathological conditions. For example, each 10-fold increase in the concentrations of superoxide and nitric oxide increases the rate of peroxynitrite formation by 100-fold. Peroxynitrite is produced by macrophages when they are activated by an immunological challenge [21], and it has been shown recently to be a major cytotoxic agent produced by inflammatory cells of the immune system [22]. It has been implicated in stroke [23, 24], heart disease [25] and immune complex-stimulated pulmonary edema [26].

Peroxynitrite is also produced by u.v. irradiation of solid potassium nitrate, imparting a stable yellow colour to such crystals [27]. Formation of peroxynitrite by exposure of nitrate in the Martian soil to u.v. light may have accounted for the spuri-

ous results from the Viking missions to detect life on Mars [28].

Peroxynitrite is 36 kcal/mol higher in energy than its isomer nitrate (NO\textsubscript{3}\textsuperscript{−}) and is a strong one-

electron oxidant with a reduction potential of 1.4 V at pH 7.0 [20]. It decomposes at physiological pH to form a species with the reactivity of hydroxyl radical (HO\textsuperscript{•}) [29]:

\[
\text{ONOO}^{−} + H^{+} \leftrightarrow \text{HOONO} \leftrightarrow \text{HO}^{•} + \text{NO}_2^{•} \tag{3}
\]

We have placed the hydroxyl radical and nitrogen dioxide in quotes because peroxynitrite does not appear to separate completely into free hydroxyl radical and nitrogen dioxide, but rather reacts as an activated complex. This energetic intermediate may be derived by bending of the N–O–O angle, and stretching of the O–O bond of trans-peroxynitrous acid appears to form a species resembling hydroxyl radical and nitrogen dioxide joined by a weak O–O bond [20].

The formation of hydroxyl radical-like oxidant is only one of several cytotoxic reactions carried out by peroxynitrite. Indeed, other direct oxidative pathways may be more important determinants of peroxynitrite toxicity. For example, more nitrogen dioxide is formed during peroxynitrous acid decomposition in the presence of hydroxyl radical scavengers; this slightly increases the bactericidal activity of peroxynitrite [30]. Peroxynitrite initiates lipid peroxidation [31] and reacts directly with sulphhydryl groups at 1000-fold greater rate than hydrogen peroxide at pH 7.4 [32]. It has been shown to inactivate sodium channels from epithelial vesicles [33]. In addition, peroxynitrite reacts with transition metals to form a powerful nitrating agent with a reactivity suggestive of the nitronium ion (NO\textsuperscript{2+}) [34].

The potential toxicity of peroxynitrite has been called into question because we have routinely used 100–250 \(\mu\text{M}\) concentrations of this oxidant \textit{in vitro}. It should be borne in mind that toxicity of a compound is proportional both to its concentration and the time of exposure [33]. Peroxynitrite decomposes rapidly at physiological pH, with a half-life of about 1 s in phosphate buffer (Figure 1) and will completely decompose in a few seconds. Thus, it is necessary to express toxicity as a function of net exposure, which is the integral of concentration versus time. We have found that the LD\textsubscript{50} for killing of \(E\text{. } escherichia\text{ } coli\) was 250 \(\mu\text{M}\) peroxynitrite, whereas 250 \(\mu\text{M}\) hydrogen peroxide over a 10 min period was not bactericidal [30]. The net exposure to peroxynitrite (the area under the curve in Figure 1) is substantially smaller than hydrogen peroxide,
even though it is far more toxic. On a concentration basis, peroxynitrite is more toxic at pH 7.4 than at 5.0. When the faster rate of decomposition of peroxynitrite at acidic pH is taken into account, there is no difference in toxicity at the two concentrations [30].

Peroxynitrite reacts with superoxide dismutase

Peroxynitrite reacts with the active site of superoxide dismutase, resulting in the production of a species with the reactivity of the toxic nitronium ion (NO$_2^+$). This was detected by nitrination of the sole tyrosine on bovine Cu,Zn-superoxide dismutase (SOD) [34, 35]. The reaction is catalytic and the enzyme is not inactivated by the reaction with peroxynitrite. We have proposed that peroxynitrite is attracted by the same electrostatic field that draws superoxide anion into the active site [36]. Removal of the copper by reduction with borohydride and dialysis against 50 mM KCN prevented nitrotyrosine formation, while restoration of copper to SOD restored full superoxide-scavenging and nitrating activity. Thus, copper in the active site of SOD was necessary for this reaction. We have proposed that peroxynitrite forms a transient cuprous adduct as follows:

$$\text{SOD–Cu}^{2+} \cdot \cdot \cdot \text{OO}–\text{N}^\cdot \cdot \cdot \text{O} \rightarrow$$

$$\text{SOD–Cu}^+ \cdot \cdot \cdot \cdot \text{O} \cdot \cdot \cdot \text{N}^\cdot \cdot \cdot \cdot \text{O}$$  (4)

This intermediate complex may donate nitronium ion to phenolics to form nitrophenols:

$$\text{SOD–Cu}^+ \cdot \cdot \cdot \cdot \text{O} \cdot \cdot \cdot \text{N}^\cdot \cdot \cdot \cdot \text{O} \rightarrow$$

$$\text{SOD–Cu}^{2+} + \text{OH}^– + \text{NO}_2^- + \text{phenol} \rightarrow$$

After releasing hydroxide ion, native SOD is regenerated.

Cu,Zn-superoxide dismutase acts catalytically and is not inactivated as a result of its reaction with peroxynitrite. SOD catalyses the nitrination of a wide range of phenolics, including tyrosines in lysozyme and histone, at a reaction rate of $10^7$ M$^{-1}$ s$^{-1}$. Thus, intravenously administered SOD to ischaemic animals may catalyse toxic nitrating reactions in ischaemic tissue, leaving specific and sensitive footprints for measuring peroxynitrite in vivo.

Macrophages produce peroxynitrite

The synthesis of nitric oxide from l-arginine contributes to the cytotoxicity of macrophages [37, 38] and fluids from macrophage-containing tumours are deficient in arginine [39]. Activated macrophages also can produce superoxide [40] with more superoxide being detectable when nitric oxide synthesis is inhibited. Conversely, SOD increases the amount of measurable nitric oxide released from these macrophages [41]. These results suggest that peroxynitrite is being produced by activated macrophages.

We have used the SOD-catalysed nitrination of a tyrosine analogue to measure peroxynitrite production from activated rat alveolar macrophages [21]. Phenolic nitrination was only observed when the macrophages were treated with phorbol esters to stimulate superoxide formation, even though the freshly isolated rat macrophages produced 60% as much nitric oxide as phorbol ester-activated macrophages. The rate of peroxynitrite formation was estimated to be 0.1 nmol 10$^6$ cells$^{-1}$ min$^{-1}$. Three other independent but indirect estimates of peroxynitrite formation by macrophage were consistent with the SOD-based measurement. First, inhibition of nitric oxide synthesis with methylarginine increased the amount of superoxide detected by the SOD-inhibitable cytochrome c reduction by 0.12±0.02 nmol 10$^6$ cells$^{-1}$ min$^{-1}$. Second, the stable decomposition products of nitric oxide–nitrite (NO$_2^-$) and nitrate (NO$_3^-$) accumulated at a rate of 0.10±0.01 nmol 10$^6$ cells$^{-1}$ min$^{-1}$ in activated macrophages. Third, the percentage of nitrate relative to nitrite increased from 30% to 67% after phorbol ester treatment. Rat alveolar macrophages produce substantial amounts of peroxynitrite, which may reach micromolar concentrations in the vicinity of the macrophage.
**The complications of inhibiting nitric oxide synthesis**

How do we assess the relative importance of nitric oxide in pathology? Given the numerous well-established deleterious effects of oxygen free radicals in vivo, we often lose sight of the importance of oxygen for the maintenance of life. For example, reperfusion with oxygen in ischaemia is necessary at some point if a tissue is to be restored to normal function. A parallel situation has arisen in understanding the contributions of nitric oxide to decrease processes. Simply inhibiting nitric oxide synthesis has often been shown to enhance injury in septic shock and myocardial ischaemia in some laboratories and to provide substantial protection in others. For example, when administered nitroarginine just prior to the initiation of cerebral ischaemia in a rat middle cerebral artery occlusion focal stroke model and found substantial mortality. Nowicki et al. [42] showed that post-administration of nitroarginine was protective in a mouse middle cerebral artery occlusion model. We then administered nitroarginine just at the time of reperfusion and found a substantial reduction of infarct volume [43]. We believe that the discrepancy between pre- and post-ischaemic treatment with nitroarginine resulted from the profound physiological effects of inhibiting nitric oxide synthesis. Nitroarginine results in a 40% reduction of cerebral blood flow in control rats, which itself will substantially increase the ischaemic area in focal stroke models dependent upon collateral circulation. The protection provided by administration at the time of ischaemia may be due to the deleterious effects of nitric oxide being prevented. We have shown previously that SOD provided similar protection to that achieved by post-treatment with nitroarginine, again suggesting that both nitric oxide and superoxide are required for injury.

A further complication of inhibiting nitric oxide synthesis with nitroarginine and methylarginine relates to the enhancement of neutrophil adherence to ischaemic tissue. In the heart, administration of nitric oxide or nitrovasodilators into the arterial supply of ischaemic heart improves myocardial function [44]. On the other hand, inhibition of endogenous nitric oxide synthesis with nitroarginine or treatment with free radical scavengers can protect against hypoxic myocardial injury [25]. Neutrophils have a well-established role in propagating myocardial ischaemic injury, and nitrovasodilators may be reducing the adhesion of neutrophils and platelets to ischaemic endothelium. Kubes and Granger [45] have shown recently that inhibition of nitric oxide synthesis greatly increases the adhesion of neutrophils to ischaemic endothelium in the cat intestine. Nitric oxide has a well-established role in reducing platelet–platelet aggregation as well as adherence to endothelium [46]. Careful evaluation of the physiological effects of nitric oxide as well as a better understanding of its reactive chemistry will be essential for elucidating its role in vivo.

This work was supported by NIH grants HL46407 and NS 24338. J.S.B. is an Established Investigator of the American Heart Association.

Cerebral ischaemia, free radicals and antioxidant protection

Edward D. Hall

CNS Diseases Research and Reactive Oxygen Program Team, The Upjohn Company, Kalamazoo, MI 49001, U.S.A.

Introduction

The role of oxygen radicals and membrane lipid peroxidation in the pathophysiology of central nervous system ischaemia has become a major focus of research. While the provision of direct and unequivocal biochemical evidence has been hampered by methodological difficulties, recent biochemical, physiological and pharmacological approaches have strongly suggested a critical role for oxygen radicals in post-ischaemic neuronal degeneration [1–5]. Potential sources of oxygen radicals following ischaemic injury to the brain or spinal cord include the enzyme xanthine oxidase, arachidonic acid metabolism, catecholamine oxidation, amine oxidase activity, mitochondrial leakage, and activated white blood cells. The primary molecular target for oxygen radical damage appears to be membrane polyunsaturated fatty acids (i.e., lipid peroxidation), although oxidative damage to proteins may also be important.

Radical formation and lipid peroxidation are catalysed by free iron released from either of the iron storage proteins, transferrin and ferritin, a haemoglobin. Tissue acidosis is also a catalyst for iron release and lipid peroxidation. Membrane lipid peroxidation can contribute to injury-induced fatty acid release and calcium influx. In relation to the important role of calcium in secondary neural damage, lipid peroxidation can serve as an amplifier by increasing membrane permeability and/or impairing calcium extrusion mechanisms and mitochondrial sequestration [2]. Free radicals have also been demonstrated to facilitate the release of excitotoxic amino acid neurotransmitters [6]. Moreover, excitotoxic neuronal damage is likely to involve a free radical process as a downstream event in its mechanistic cascade [7]. Indeed, free radical-induced lipid peroxidation may be the final

Abbreviation used: MCA, middle cerebral artery.