Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and the ugly

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Beckman, Joseph S., and Willem H. Koppenol. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and the ugly. *Am. J. Physiol.* 271 (*Cell Physiol.* 40): C1424–C1437, 1996.—Nitric oxide contrasts with most intercellular messengers because it diffuses rapidly and isotropically through most tissues with little reaction but cannot be transported through the vasculature due to rapid destruction by oxyhemoglobin. The rapid diffusion of nitric oxide between cells allows it to locally integrate the responses of blood vessels to turbulence, modulate synaptic plasticity in neurons, and control the oscillatory behavior of neuronal networks. Nitric oxide is not necessarily short lived and is intrinsically no more reactive than oxygen. The reactivity of nitric oxide per se has been greatly overestimated in vitro, because no drain is provided to remove nitric oxide. Nitric oxide persists in solution for several minutes in micromolar concentrations before it reacts with oxygen to form much stronger oxidants like nitrogen dioxide. Nitric oxide is removed within seconds in vivo by diffusion over 100 µm through tissues to enter red blood cells and react with oxyhemoglobin. The direct toxicity of nitric oxide is modest but is greatly enhanced by reacting with superoxide to form peroxynitrite (ONOO⁻). Nitric oxide is the only biological molecule produced in high enough concentrations to out-compete superoxide dismutase for superoxide. Peroxynitrite reacts relatively slowly with most biological molecules, making peroxynitrite a selective oxidant. Peroxynitrite modifies tyrosine in proteins to create nitrotyrosines, leaving a footprint detectable in vivo. Nitration of structural proteins, including neurofilaments and actin, can disrupt filament assembly with major pathological consequences. Antibodies to nitrotyrosine have revealed nitration in human atherosclerosis, myocardial ischemia, septic and distressed lung, inflammatory bowel disease, and amyotrophic lateral sclerosis.

superoxide dismutase; toxicity; neuronal networks

NITRIC OXIDE SERVES CROSSED purposes. It is a ubiquitous intercellular messenger in vertebrates, modulating blood flow, thrombosis, and neural activity. The production of nitric oxide is also important for nonspecific host defense, helping to kill tumors and intracellular pathogens. Cytotoxicity is often directly attributed to nitric oxide, which is erroneously described in the biological literature as short lived and highly reactive. Yet, how can nitric oxide be produced by neurons for 80 years without overt toxicity but also be highly damaging when produced in 10- to 100-fold higher concentrations after only a few minutes during pathological events like cerebral ischemia? In this review, we explore why nitric oxide itself is far less reactive and toxic at biologically relevant concentrations than in vitro experiments suggest. The rates of oxidation of organic molecules by nitric oxide under anaerobic conditions are about the same as the rates of oxidation initiated by molecular oxygen (48, 49, 100). Nitric oxide is so useful as an intracellular messenger because it can diffuse through most cells and tissues with little consumption or direct reaction (55, 105). The rapid diffusion of nitric oxide between cells is crucial for understanding its biological activity. The toxicity of nitric oxide is more likely to result from the diffusion-limited reaction of nitric oxide with superoxide (O_2^-) to produce the powerful and toxic oxidant peroxynitrite (ONOO⁻). In effect, peroxynitrite is a binary toxin assembled spontaneously whenever nitric oxide and superoxide are produced together.

The regulation and synthesis of nitric oxide by mammalian cells have been the subject of many excellent reviews (73), as have the physiological and pathological

effects of nitric oxide (26, 68). We instead focus on how the chemistry of nitric oxide affects its biological actions, hopefully illuminating some of the subtle problems of interpretation encountered with in vitro experiments.

Nitric oxide is an uncharged molecule composed of seven electrons from nitrogen and eight electrons from oxygen (Fig. 1). Because orbitals can contain a maximum of two electrons, the highest occupied orbital in nitric oxide must contain an unpaired electron. However, nitric oxide is not highly reactive simply because it is a free radical. Molecular oxygen contains two unpaired electrons and, clearly, tissues survive containing between 20 and 200 µM dissolved oxygen. The majority of biological molecules contain bonds filled with two electrons, which makes them unreactive with nitric oxide. Nitric oxide only reacts rapidly with a select range of molecules that have orbitals with unpaired electrons, which are typically other free radicals, and with transition metals like heme iron. The rates for many reactions involving nitric oxide are known, which permits us to focus on a few reactions fast enough to predominate under biologically relevant conditions. With the use of reaction rates as a guide, the biological chemistry of nitric oxide can be simplified in a reasonable approximation to just three main reactions (Fig. 2): its activation of guanylate cyclase, responsible for signal transduction; its destruction by reaction with oxyhemoglobin (24, 34); and its transformation to $ONOO^-$ by the reaction with O_2^-. Nitric oxide will rapidly combine with other free radicals (75, 85), although our knowledge of these reactions is in its infancy. It is an oversimplification to limit ourselves to just three reactions, but the key point is that nitric oxide does not react rapidly with most biological molecules at the dilute concentrations produced in vivo. In particular, the reaction with oxygen to form nitrogen dioxide is not likely to be significant in vivo, because

$$\cdot O\equiv O\cdot$$
Oxygen

$$\cdot N\equiv O \;\rightleftharpoons\; 1/2\; O=N-N=O$$
Nitric oxide   Dinitrogen dioxide

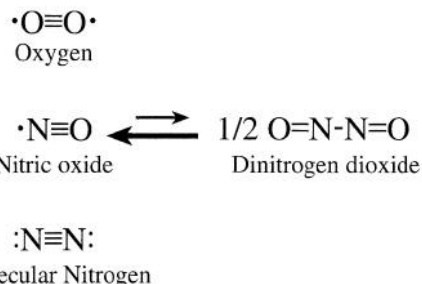$$:N\equiv N:$$
Molecular Nitrogen

Fig. 1. Why is nitric oxide a free radical? Even in saturated 2 mM solutions, nitric oxide remains as a free radical rather than dimerizing to form dinitrogen dioxide. The reason is that no net bonds are made when nitric oxide dimerizes, so entropy at room temperature favors 2 separate nitric oxides. Nitric oxide is intermediate between nitrogen, with a bond order of 3, and oxygen, with a bond order of 2. The unpaired electron on nitric oxide exists in an antibonding π orbital, which weakens the triple bond by 0.5, resulting in an overall bond order of 2.5. Oxygen has 2 unpaired electrons in separate antibonding orbitals, which weakens the triple bond by 2 times one-half bonds to give a bond order of 2. When nitric oxide dimerizes, the bond order is 5, which is no greater than 2 separate nitric oxide molecules. The dimer can be trapped at liquid nitrogen temperatures as a pale blue solid but completely dissociates at room temperature.
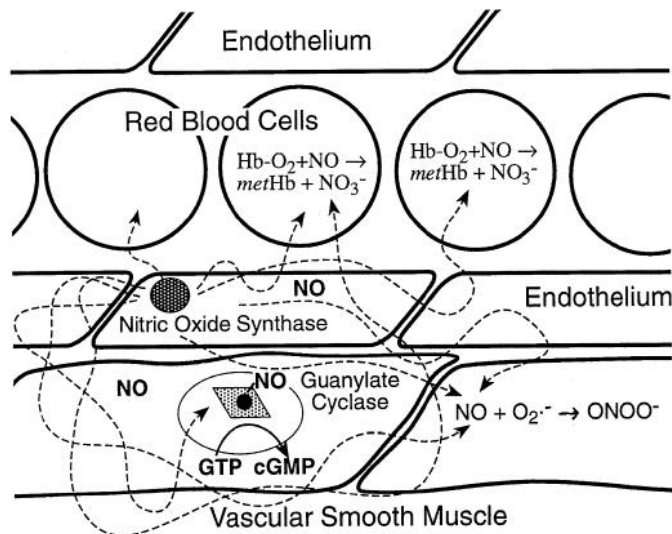

Fig. 2. Principal reactions of nitric oxide in tissue. Nitric oxide produced in an endothelial cell will diffuse out far faster than it will react with most intracellular components. Nitric oxide binds to and activates soluble guanylate cyclase. If it diffuses into a red blood cell, nitric oxide will be eliminated by oxyhemoglobin ($Hb-O_2$) to form nitrate. The reaction with superoxide to form peroxynitrite is normally limited by the micromolar concentrations of superoxide dismutase in cells. metHb, methemoglobin.

diffusion to blood vessels is far more rapid and removes nitric oxide by reaction with oxyhemoglobin in red blood cells.

Nitric oxide can now be monitored directly in submicromolar concentrations, making it easy to demonstrate that nitric oxide is stable at biologically relevant concentrations in both buffers and hemoglobin-free tissue homogenates. We have used a simple method that allows nitric oxide dissolved in solutions to diffuse across a thin hydrophobic tubing into a stream of helium, leading to a chemiluminescent nitric oxide detector (Fig. 3; Ref 5). This method was originally reported by Lewis et al. (59) for use with mass spectrometers. Similar results can be obtained by using commercially available nitric oxide electrodes, although their response time can be much slower and electrodes are
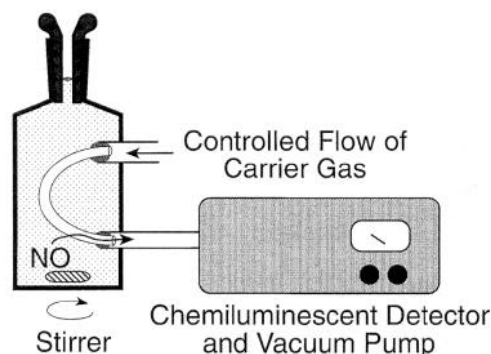

Fig. 3. Nitric oxide can be readily monitored in solution by allowing nitric oxide to diffuse through a microporous membrane into a stream of helium gas. The gas is fed to a commercial chemiluminescent nitric oxide detector (Antek Instruments, Houston, TX). Submicromolar concentrations of nitric oxide can be continuously monitored in this manner.

subject to more interference. Approximately 20% of 1.2 µM nitric oxide remained after incubation for 10 min in Krebs-Henseleit buffer saturated with 95% oxygen, which is still 50-fold greater than needed to relax a vascular ring in vitro. (Fig. 4). The consumption of nitric oxide by the measuring device was the dominant cause for the decrease of nitric oxide in this system, rather than the reaction of nitric oxide with oxygen. The addition of copper, zinc superoxide dismutase had only a slight effect on the loss of nitric oxide. The addition of free oxyhemoglobin or a few red blood cells caused the rapid destruction of nitric oxide. Oxyhemoglobin rapidly combines with nitric oxide to yield methemoglobin and nitrate (24). When enough nitric oxide is added to react with all of the oxyhemoglobin, then nitric oxide is as stable in the presence of methemoglobin as in buffer alone (Fig. 4). Plasma itself does not consume nitric oxide (not shown).

The diffusion of nitric oxide is sufficiently rapid that nitric oxide produced in tissues is more likely to diffuse into the vascular compartment and be destroyed by hemoglobin than to react with the normal constituents in tissues. Clearly, oxygen is able to diffuse over 100 µm from a blood vessel to reach mitochondria in tissues. Therefore, nitric oxide, which has a 1.4-fold larger diffusion coefficient at 37°C than oxygen (64, 104), can diffuse over 100 µm back to a blood vessel within a few seconds.
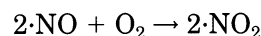
FORMATION OF NITROGEN DIOXIDE

The reputation of nitric oxide as being a highly reactive molecule comes from two observations: the rapid formation of nitrogen dioxide when high concentrations of nitric oxide mix with oxygen, and the short

Fig. 4. Duration of 1 µM nitric oxide in Krebs-Henseleit buffer saturated with 95% oxygen. The majority of the nitric oxide is being consumed by diffusion into the membrane and measurement by the nitric oxide analyzer rather than by reaction with oxygen or buffer components. Nitric oxide is immediately consumed by the addition of 0.5 µM hemoglobin. Addition of superoxide dismutase (SOD) has little effect on the disappearance of nitric oxide under these conditions.

Table 1. *Half-life of nitric oxide due to reaction with oxygen predicted by Eq. 1*

NO, µM	Half-Life, s
1,000	0.56
100	5.6
10	56
1	560
0.1	5,600
0.01	56,000

The concentration of oxygen is assumed to be 225 µM for water saturated with room air.

apparent half-life of nitric oxide in perfusion cascades. If a tank of pure nitric oxide is allowed to leak into air, a cloud of lethal and highly reactive orange-brown nitrogen dioxide forms by the following reaction

$$2 \cdot NO + O_2 \rightarrow 2 \cdot NO_2$$

Nitrogen dioxide is a strong and toxic oxidant, but the rate of its formation depends on the square of nitric oxide concentration ([NO]), because two nitric oxide molecules must collide with each oxygen molecule. The rate is given by

$$\frac{d[NO]}{dt} = 4k_3[O_2][\cdot NO]^2 \qquad (1)$$

where k_3 is 2×10^6 $M^{-2} \cdot s^{-1}$ (27). The factor of 4 comes from the rapid attack of the two nitrogen dioxides on two additional nitric oxides to form dinitrogen trioxide (N_2O_3), a strong one- and two-electron oxidant. The N_2O_3 can also react with water to give two nitrite molecules

$$NO + NO_2 \leftrightarrow N_2O_3 + H_2O \rightarrow 2NO_2^- + 2H^+$$
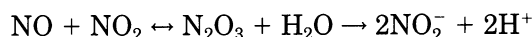
Consequently, nitrite is the major decomposition product of nitric oxide in simple buffers (44). When nitric oxide is added to a simple buffer containing bovine serum albumin or other proteins, the apparent half-life of nitric oxide increases, because nitrogen dioxide reacts directly with the protein rather than with a second nitric oxide.

A saturating solution of nitric oxide is ~2 mM and has a half-life of <1 s in water. The half-life of nitric oxide determined by *Eq. 1* is $1/(k_3[O_2][\cdot NO])$. Consequently, half-life is not a constant value and is inversely proportional to the concentration of nitric oxide, so the half-life becomes much longer as nitric oxide becomes more dilute (Table 1). Physiological solutions are 1,000–200,000 times more dilute [ranging from a maximum of ~4 µM, the amount measured during cerebral ischemia (61), to 5 nM, the minimum concentration required to activate guanylate cyclase]. Thus the reaction rate slows down by a factor of 1,000,000–400,000,000 when nitric oxide is diluted to physiologically relevant concentrations. The half-life of 5 nM nitric oxide is >100,000 s or 70 h. Clearly, the low probability of two nitric oxide molecules encountering each other in a biological system makes the formation of nitrogen dioxide extremely slow under physiological conditions, as shown by the long half-life of nitric oxide in Fig. 4.
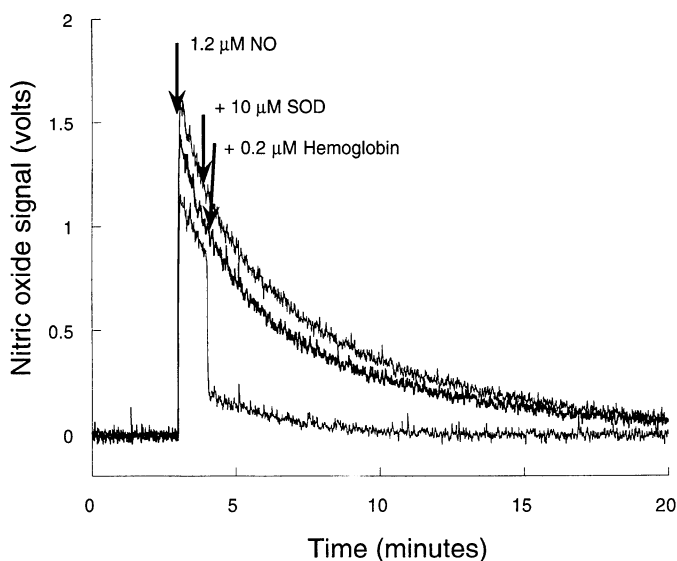
Atmospheric chemists have long known that the oxidation of nitric oxide is an insignificant process, even on the most polluted days over Los Angeles. In air pollution studies, a far more significant reaction is the reaction of the conjugate acid of superoxide ($HO_2\cdot$) to form peroxynitrous acid that immediately decomposes in the gas phase to hydroxyl radical and nitrogen dioxide (11, 14). The solution chemistry of peroxynitrite is a bit more complex (53, 79).

Clinicians are ventilating patients with micromolar concentrations of nitric oxide in air for weeks to combat pulmonary hypertension (8, 28, 30), effectively treating a formerly intractable clinical problem without causing apparent harm. Concentrations of nitric oxide that cause vasorelaxation are not highly toxic.

THE "SHORT" HALF-LIFE OF NITRIC OXIDE IN PERFUSION CASCADES

In the original bioassay systems, the lifetime of the endothelium-derived relaxing factor (EDRF) was only a few seconds in oxygenated Krebs-Henseleit buffers. This was originally attributed to the direct reaction with oxygen to form nitrogen dioxide, which is clearly too slow to be significant at physiological concentrations. Superoxide formed in the buffer is more likely to account for the short half-life of nitric oxide in vitro. Superoxide dismutase (15–100 U/ml) approximately doubles the apparent half-life of EDRF, strongly suggesting that superoxide contributes to the short biological half-life of nitric oxide (76, 84). The Krebs-Henseleit buffer used in perfusion cascade bioassay systems provides prime conditions for contamination artifacts that have plagued and fascinated free radical chemists for decades. It contains 11 mM glucose, is contaminated with trace iron and copper, is bubbled with 95% oxygen ($\sim$1 mM), and is incubated under the ultraviolet radiation of fluorescent lights, all of which contribute to the formation of superoxide.

The reaction rate between superoxide and nitric oxide occurs at the near-diffusion-limited rate of 6.7×10^9 $M^{-1}\cdot s^{-1}$ (43), so that nearly every collision between superoxide and nitric oxide results in the irreversible formation of peroxynitrite. The shortest half-life of nitric oxide measured in perfusion cascades is 3–6 s. Only 5 nM of nitric oxide is required to relax vascular rings, which can be readily destroyed by superoxide formation by glucose autoxidation in Krebs-Henseleit buffer. In the experiments shown in Fig. 4, 1 µM nitric oxide was relatively stable in Krebs-Henseleit buffer. Under these conditions, the rate of glucose autoxidation was too slow to consume the amount of nitric oxide injected and thus did not appear to affect the stability of nitric oxide.

Why does superoxide dismutase not fully protect nitric oxide from inactivation in perfusion cascades? Because of the diffusion-limited reaction between superoxide and nitric oxide, 15–100 units of superoxide dismutase are not sufficient to "sop up" all of the superoxide. A unit of superoxide dismutase activity is defined as inhibiting the rate of 10 µM cytochrome c reduction by 50% in a 3-ml volume. Superoxide reacts

with nitric oxide 7,000 times faster than cytochrome c. Superoxide dismutase at the concentrations used would be only partially effective at scavenging superoxide, because it is less efficient than nitric oxide as a scavenger of superoxide. In perfusion cascades, few could afford to use the grams of superoxide dismutase that would be necessary to fully block the reaction of nitric oxide and superoxide.

A more subtle effect is illustrated by measuring nitric oxide released from the decomposition of 3-morpholinosydnonimine (SIN-1), a nitrovasodilator that can simultaneously generate superoxide and nitric oxide (20). No nitric oxide can be detected from 1 mM SIN-1 until superoxide dismutase is added (Fig. 5). The recovery of nitric oxide increases as more superoxide dismutase is added, with $\sim$5,000 units of superoxide dismutase (which is $\sim$30 µM) being required to maximize the recovery of nitric oxide (Fig. 5). Essentially identical results have been observed by Mayer et al. (63) with purified nitric oxide synthase. Because of autoxidation of biopterin to form superoxide, enormous amounts of superoxide dismutase must be added to maximize the detection of nitric oxide. The reason is simple: little else other than superoxide in the system can remove nitric oxide. With each 10-fold increase of superoxide dismutase, the concentration of superoxide decreases 10-fold. The steady-state concentration of nitric oxide then increases 10-fold to the point where nitric oxide again competes with the additional superoxide dismutase for superoxide. The rate of peroxynitrite formation is determined by the product of nitric oxide and superoxide concentrations, so peroxynitrite formation remains approximately the same. As the Red Queen admonished Alice in Wonderland, you must run as fast as you can to stay right where you are. Only when the nitric oxide concentration rises to the micromolar range will the reaction of nitric oxide with oxygen to give nitrogen dioxide begin to compete with superoxide. In vivo, the constant removal of nitric oxide
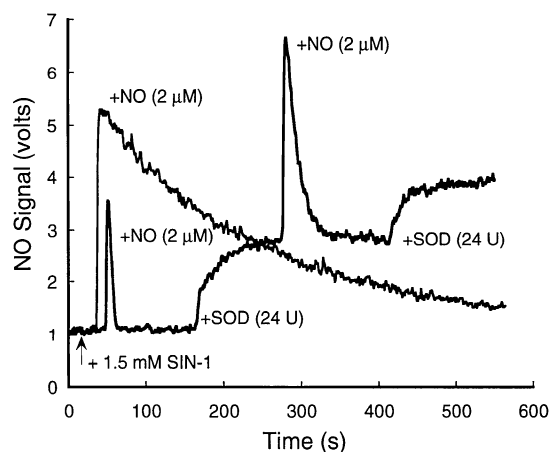
Fig. 5. The nitrovasodilator 3-morpholinosydnonimine (SIN-1) will reduce oxygen to superoxide and release nitric oxide in simple phosphate buffer. No nitric oxide is detectable from 1 mM SIN-1 until SOD is added. Approximately 5,000 U/ml of SOD (1.0 mg/ml) is required to maximize the detectable nitric oxide. A secondary rapid reaction is occurring between nitric oxide and peroxynitrite, which appears to form nitrite and nitrogen dioxide.

by hemoglobin and other reactions will allow superoxide dismutase to successfully reduce peroxynitrite formation by limiting the accumulation of nitric oxide. However, in vitro, the addition of superoxide dismutase does not necessarily slow the production of peroxynitrite, unless vast amounts are added.

REMEMBERING THE KITCHEN SINK

The steady-state concentration of any molecule is a balance between the rate of formation and the rate of removal, much as the level of water in a sink is determined by how fast water is poured into the sink and how fast it is drained away (Fig. 6). Few studies directly measure the concentration of nitric oxide and most assume that nitric oxide must be continuously added because of its short half-life. Without oxyhemoglobin or some other drain to remove nitric oxide, the concentration of nitric oxide will rise to the micromolar range, at which nitrogen dioxide will be quantitatively formed. The steady-state concentration of nitric oxide in a simple buffer system will be given by

$$[NO] = \sqrt{\frac{k_f}{4k_3[O_2]}}$$

where k_f is the rate of nitric oxide loss and $4k_3$ has been consistently determined to be $6-8 \times 10^6 \ M^{-1} \cdot s^{-1}$ (27). In a simple buffer system, the half-life of 1 μM nitric oxide is 560 s. Clearly, nitric oxide hangs around in these simple in vitro systems far longer than it will in vivo! Given long enough, nitric oxide will react with oxygen to form nitrogen dioxide and thus appear to be reactive.

When either hemoglobin or myoglobin is present, even when packaged in red blood cells located several hundred microns away in a blood vessel, the half-life of nitric oxide will be much shorter. The half-life is largely determined by the time needed to diffuse to a blood vessel. The steady-state concentration is simply the ratio of the rate of formation over the rate of nitric oxide
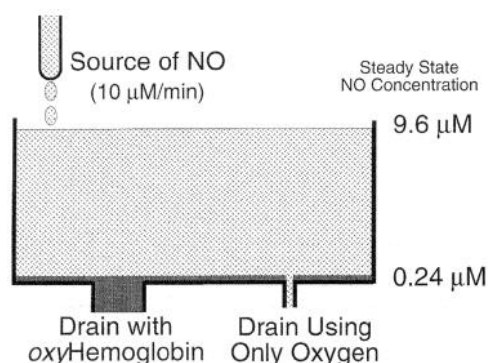

Fig. 6. When evaluating toxicity of either nitric oxide or oxygen radicals, drains that remove these compounds must also be considered. Without a drain provided, steady-state concentrations will rise, much as a kitchen sink will flood if the drain is closed. Concentration of nitric oxide is illustrated for a source producing 10 nmol·min^{-1}·ml^{-1} of nitric oxide, when the nitric oxide is removed by 1) reaction with oxygen or 2) diffusion with a half-life of 1 s to a red blood cell.

decomposition. Expressing the rate of decomposition in terms of half-life gives

$$[NO] = \frac{k_f \times t_{\frac{1}{2}}}{0.693}$$

where 0.693 is the natural log of 2. Suppose that we assume that the biological half-life is 1 s, a bit longer than measured in heart (51), and that a cell is producing 1 μM nitric oxide per minute, which would be a high rate for a fully activated macrophage. Under these conditions, the steady-state concentration of nitric oxide will be 0.24 μM and <3 of 10,000 nitric oxide molecules will react with oxygen to form nitrogen dioxide. The nitric oxide concentration would be 9.6 μM if oxygen were the only route of removal. Clearly, only minuscule amounts of the nitric oxide are consumed to produce nitrogen dioxide when other means are present to remove nitric oxide. In vitro experiments vastly overestimate the cytotoxic potential and the chemical reactivity of nitric oxide by ignoring the drains that remove nitric oxide.

NITRIC OXIDE DONORS

In most experiments studying the reactivity of nitric oxide in vitro, generating systems are used to continuously produce nitric oxide on the assumption that nitric oxide is so unstable that it must be continuously supplied. A variety of nitric oxide donors are utilized to slowly release nitric oxide, and any observed effects are usually ascribed to nitric oxide itself. However, the reactivity of nitric oxide donors toward most biological molecules is often greater than that of nitric oxide. For example, sodium nitroprusside attacks thiols and releases cyanide (2). Not surprisingly, millimolar concentrations of a compound that releases up to five molecules of cyanide can also inhibit cell proliferation. Sodium nitroprusside also can form complexes with many metalloproteins, including superoxide dismutase, through processes that are not yet understood (66). Nitrosothiols release only minute amounts of nitric oxide in metal-free buffers that are protected from light. However, nitrosothiols can be much better thiol-modifying agents than nitric oxide itself. Pryor et al. (78) have shown that the oxidation of cysteine at pH 7.4 by nitric oxide takes 6 h and the products are nitrous oxide (N_2O) and nitrite, not nitrosothiol. Wink et al. (102) have also found that nitric oxide modifies thiols only after oxidation to secondary species and not under anaerobic conditions. The nitrovasodilator SIN-1 in simple buffer solutions releases no detectable nitric oxide in the absence of superoxide dismutase because all of the nitric oxide is converted to peroxynitrite (Fig. 5). In complex media such as cell culture media or plasma, SIN-1 appears to reduce components other than oxygen and can release detectable amounts of nitric oxide in the absence of superoxide dismutase. Consequently, SIN-1 is an effective nitrovasodilator in vivo but cannot be assumed to be either a nitric oxide or peroxynitrite generator without consideration of the media.

A more subtle artifact of prolonged activation of soluble guanylate cyclase is the crossover of guanosine 3',5'-cyclic monophosphate (cGMP) for activating adenosine 3',5'-cyclic monophosphate (cAMP)-dependent processes (16). When high concentrations of nitric oxide are applied to cells for long periods of time, the concentration of cGMP can rise to high enough levels to activate cAMP-dependent processes. Activation of cAMP-dependent kinase by cGMP has been shown to account for the inhibition of myocyte proliferation in vitro (16).

NITROSOTHIOLS

Nitrosothiols are found in vivo in <1 µM concentrations (90) and have been proposed to be a major form of EDRF (72). They are vasodilatory, and the nitrosothiols found naturally in plasma have half-lives of ~40 min (90). The half-lives of nitrosothiols are strongly influenced by other reactants, such as the concentration of free thiols or the presence of transition metals. Nitrosothiols in serum are not inactivated by hemoglobin in red blood cells.

Nitric oxide is sometimes viewed as being rapidly interchangeable with nitrosonium ion (NO^+). Because of the high 1.2-V potential for oxidation of nitric oxide (53), nitric oxide does not directly nitrosate organic molecules without a strongly oxidizing cofactor to accept an electron and balance the following reaction

$$\cdot NO + RSH \rightarrow RSNO + H^+ + e^-$$

Common electron acceptors that will participate in nitrosating reactions include nitrogen dioxide and transition metals. For example, nitric oxide bound to ferric heme iron can nitrosate phenolics, thiols, and secondary amines (98). Free nitrosonium itself will not exist long in solution because it will react immediately with water (55 M) to form nitrite. However, the nitrosonium ion moiety can be transferred between biological molecules, and thiols are probably the most common transfer agents in vivo. A large literature exists concerning nitrosoamines because of their carcinogenic potential.

Thiols such as glutathione are widespread antioxidants and are continuously being oxidized to thiyl radicals. A rapid and biologically likely reaction to form nitrosothiols in vivo is the radical-radical combination

$$RS\cdot + \cdot NO \rightarrow RSNO$$

If the half-life of nitric oxide in vivo is between 1 and 10 s, then it would need to be produced at a rate 240–2,400 times faster than nitrosothiols (assuming a 40-min half-life) to achieve the same steady-state concentration in vivo. With a half-life of 40 min, relatively little of the nitric oxide produced in vivo would need to react with a thiyl radical to generate the observed amounts of nitrosothiols in serum.
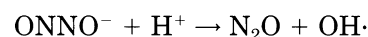
NITROXYL ANION

Nitroxyl anion has been suggested to be a potential EDRF (31, 71), but it should be remembered that

nitroxyl anion is a reactive and short-lived species. In vitro, nitroxyl anion will react rapidly with up to two nitric oxides to form nitrous oxide and nitrite (88, 89)

$$NO^- + NO \rightarrow ONNO^-$$

$$ONNO^- + NO \rightarrow N_2O + NO_2^-$$
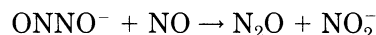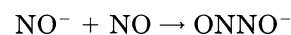
These reactions are close to diffusion limited, so nitric oxide can be rapidly scavenged by nitroxyl anion. When nitric oxide is dilute, the intermediate $ONNO^-$ can also decompose to form hydroxyl radical

$$ONNO^- + H^+ \rightarrow N_2O + OH\cdot$$

The intermediate $ONNO^-$ has many similarities with peroxynitrite, except that it will be a one-electron oxidant, whereas peroxynitrite can be both a one- and two-electron oxidant.

Nitroxyl anion reacts with molecular oxygen, which is usually present in much higher concentrations than nitric oxide, to form peroxynitrite anion (23, 42)

$$NO^- + O_2 \rightarrow ONOO^-$$
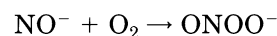
It is a useful synthetic preparation of peroxynitrite (108). The rate for this reaction has not been directly measured but is likely to be fast and will limit the lifetime of nitroxyl anion to only a few microseconds.

Peroxynitrite will cause vessel relaxation and increase cGMP in smooth muscle in concentrations that are 50- to 1,000-fold higher than nitric oxide (60, 94, 106). However, it is unlikely that either nitroxyl anion or peroxynitrite is a major form of EDRF, given their reactivity, toxicity, and modest efficacy as vasorelaxing agents.

DIFFUSION, DRAINS, AND INFORMATION

Receptors in target tissues can easily distinguish between two closely related molecules produced by distant endocrine systems, like norepinephrine and epinephrine, because of subtle differences in shape. With only two atoms, nitric oxide encodes information not by its shape but by changes in its local concentration. If nitric oxide were transported in a more stable form, the cross talk between different tissues would obliterate any information being transferred by nitric oxide. Guanylate cyclase producing cGMP is still the only well-established signal transduction mechanism for nitric oxide. Nitric oxide activates guanylate cyclase by binding to ferrous heme, which it does with a much higher affinity than oxygen (91, 92, 95). At present, the mechanism by which nitric oxide is released from guanylate cyclase is unknown, although cGMP production stops within seconds after nitric oxide is removed (33). To convey information, local concentrations of nitric oxide must be rapidly removed to prevent saturation of guanylate cyclase.

Because membranes provide no greater barrier to nitric oxide than to oxygen or carbon dioxide, diffusion away from a single cell producing nitric oxide is more significant than reactions of nitric oxide within the cell

(55). Nitric oxide will diffuse in and out of a cell membrane thousands of times in a second.

Diffusion also allows for the rapid summation of nitric oxide production by several cells within a local region. When one whispers in a lecture room, the sound dampens with the square of distance as it propagates radially away from the source. Only a few people close enough to hear the whisper will be influenced by the information communicated. In a well-designed lecture room, the sound is eventually dampened by absorbent walls, much as hemoglobin in blood vessels will capture nitric oxide diffusing through tissues. Several people scattered around a room can whisper at once without affecting each other. However, the noise level in a crowded lecture room rises dramatically when everyone speaks simultaneously. The same happens when a local group of cells is producing nitric oxide. The net concentration of nitric oxide within a cell can be much greater from the contributions of surrounding cells than from its own internal production.

Red blood cells provide a drain that creates a sharp diffusion gradient leading to the vasculature. A red blood cell can reduce the internal concentration of nitric oxide in a nitric oxide-producing cell because the oxyhemoglobin will greatly reduce the reentry of nitric oxide into the cell (55). Endothelium can make 10- to 40-fold more nitric oxide than needed to activate guanylate cyclase, as measured by microelectrodes placed against isolated cells (62), but the majority of the nitric oxide will be lost to the vascular compartment. Even after nitric oxide has diffused into the smooth muscle, it can rapidly diffuse back along the diffusion gradient to the red blood cell (Fig. 2). In the perfusion cascades originally used to assay EDRF, the excess production of nitric oxide in the absence of hemoglobin was sufficient to relax artery rings, even though they were diluted in a large volume of perfusion buffer (84).

Although the biological half-life of nitric oxide is only a scant second in vivo, 1 s is long compared with a simple neural reflex or a muscle contraction. A world class sprinter can run 100 m in 10 s, moving at least 10 m within the reported half-lives of EDRF. The shortest interval we can consciously perceive is around one-fortieth of a second. The intermediate duration of nitric oxide and its rapid diffusion through most tissues allows nitric oxide to integrate and modulate complex physiological processes. In effect, nitric oxide is the equivalent of a shock absorber that dampens the oscillations of a car driving over a bumpy road.

The modulating effects of nitric oxide are illustrated by the release of nitric oxide from endothelium in response to turbulent flow. When a distal vascular bed dilates to supply more blood flow to an actively working muscle, the upstream blood vessels must also dilate to support the increased blood flow. Otherwise, turbulence would develop whenever the Reynolds number for laminar flow is exceeded (36). In the absence of endothelium, the stress of turbulent flow would be countered by myogenic contraction that would cause a further increase in shear stress. Further constriction can amplify turbulent flow, potentially leading to a catastrophic collapse of blood flow to distal vascular beds. However, shear-induced stress from turbulence induces endothelium to synthesize nitric oxide, which causes a local relaxation of the underlying vascular smooth muscle, increases the diameter of the blood vessel, and locally restores laminar flow. The local relaxation of the underlying smooth muscle balances the myogenic responses to turbulent flow and thereby ensures a laminar distribution of blood between vessels (36).

A potential complication of nitric oxide modulation of vascular tone is the development of atherosclerosis, which tends to develop at sites of low turbulence. Macrophages accumulate in subendothelial spaces, ingesting oxidized lipoproteins to form atherosclerotic lesions. The mechanism that initially oxidizes lipoproteins has never been proven, but Graham et al. (35) and Hogg et al. (41) have shown that peroxynitrite modifies low-density lipoproteins into a form recognized by the macrophage scavenger receptor. Although the responsiveness of atherosclerotic blood vessels to endothelium-dependent relaxation is greatly diminished, such vessels show greater release of nitrite and nitrate as well as upregulation of the inducible form of nitric oxide synthase, indicating that nitric oxide is being inactivated more quickly. Superoxide dismutase improves endothelium-dependent relaxation, particularly when conjugated to polyethylene glycol or encapsulated in liposomes to increase delivery to the atheroma (70, 99), further indicating that superoxide is attacking nitric oxide to form peroxynitrite. We have found that nitrotyrosine, a marker of peroxynitrite oxidation of proteins, is widespread in human atherosclerotic lesions (7). Nitration of endogenous proteins in the vessel wall may enhance their antigenicity, favoring the recruitment of additional inflammatory cells to the vessel wall. As the atherosclerotic lesion develops, it reduces the arterial vessel diameter, thereby increasing turbulence and favoring further production of nitric oxide.

NITRIC OXIDE AS A NEUROTRANSMITTER

Although the production of nitric oxide in brain is well established, its properties are substantially different from those of most neurotransmitters. A traditional neurotransmitter like acetylcholine lasts only a few milliseconds after release from synapses, whereas nitric oxide will persist for seconds. The rapid diffusion of nitric oxide enables it to encompass regions containing up to several million synapses (105). These properties make nitric oxide more likely to serve as a feedback loop, influencing oscillatory behavior and potentially acting as a retrograde messenger (32, 69).

DIRECT TOXIC EFFECTS OF NITRIC OXIDE

Nitric oxide is reported to have a multitude of potentially toxic effects, although many of these are more likely mediated by oxidation products rather than by nitric oxide itself. Thus nitric oxide does not directly attack DNA unless it is allowed to oxidize to higher nitrogen oxides (101). For example, nitric oxide does not directly ribosylate glyceraldehyde-3-phosphate de-

hydrogenase (22). Rather, the nitrosothiols and sodium nitroprusside commonly used as nitric oxide donors react directly with a sulfhydryl anion in the active site (67). The production of nitric oxide by activated macrophages will inactivate iron/sulfur centers in tumor cell mitochondria (25, 40). Nitric oxide will also bind reversibly to iron/sulfur proteins (56), but more recent experiments suggest that the inactivation of iron/sulfur centers is mediated by peroxynitrite rather than nitric oxide (12, 39). Inactivation of aconitase by nitric oxide appears to be slow and comparable with the rate of inactivation by molecular oxygen. Activated macrophages also produce superoxide, so the inactivation of tumor cell mitochondria may well be mediated by peroxynitrite (80). Indeed, the inactivation of mitochondria by peroxynitrite in vitro is remarkably similar to the respiratory inhibition observed in cultured tumor cells caused by activated macrophages (80, 93).

Nitric oxide can reversibly inhibit enzymes containing transition metals or free radical intermediates in their catalytic cycle. Nitric oxide in micromolar concentrations will reversibly inhibit catalase and cytochrome P-450 (103). It can also inhibit ribonucleotide reductase, a critical enzyme for synthesis of DNA precursors that contains a tyrosine radical (54, 58). Inhibition of DNA synthesis by nitric oxide inhibits viral replication (50). However, the inhibition of ribonucleotide reductase is reversible and lost when nitric oxide drops less than a few micromoles per liter in concentration. Large continuous fluxes of nitric oxide are necessary to inhibit ribonucleotide reductase and are most likely to occur only with severe sepsis or in cells near activated macrophages. Enormous amounts of oxygen are required to maintain synthesis of nitric oxide in micromolar concentrations. Nitric oxide synthase requires two oxygens per nitric oxide produced. If the half-life of nitric oxide in vivo is ~1 s, then 170 nmol O_2/min would be needed per gram of tissue to maintain nitric oxide at a steady-state concentration of 1 μM nitric oxide.

Nitric oxide in the micromolar range can also reversibly inhibit cytochrome-c oxidase (15, 87), which may transiently increase the leakage of superoxide from the electron transport chain. If the internal nitric oxide concentration approaches that of mitochondrial superoxide dismutase, the superoxide would largely react with the nitric oxide and the peroxynitrite formed would irreversibly injure mitochondria (74, 81).

PEROXYNITRITE: THE UGLY SIDE OF NITRIC OXIDE

A major mechanism of injury associated with the production of nitric oxide in vivo is due to its diffusion-limited reaction with superoxide to form peroxynitrite. Superoxide is produced by the reduction of molecular oxygen by one electron, which has two unpaired electrons

$$\cdot O\text{–}O\cdot + 1e^- \rightarrow {}^-\!:O\text{–}O\cdot \ (O_2^-)$$
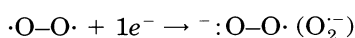
Consequently, superoxide still has one unpaired electron that rapidly combines with nitric oxide, with the release of 22 kcal/mol

$$^-\!:O\text{–}O\cdot + \cdot N\text{–}O \rightarrow {}^-\!:O\text{–}O\text{–}N\text{–}O$$
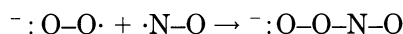
The large Gibbs energy makes the formation of peroxynitrite essentially irreversible, i.e., thermodynamically equivalent to the hydrolysis of two ATPs. Peroxynitrite is not a free radical because the unpaired electrons on superoxide and on nitric oxide have each combined to form a new chemical bond.

How can two free radicals present in low concentrations find each other in vivo? The reaction rate for the formation of peroxynitrite has recently been determined to be $6.7 \pm 0.9 \times 10^9 \ \text{M}^{-1}\cdot\text{s}^{-1}$ (43), which is approximately six times faster than the scavenging of superoxide with copper, zinc superoxide dismutase at physiological ionic strength (19, 82). Nitric oxide is the only known biological molecule produced in high enough concentrations under pathological conditions to outcompete endogenous superoxide dismutase for superoxide.

Although the concentration of superoxide is kept low by superoxide dismutase, a large flux of superoxide is produced by aerobic metabolism. Generally, 1–5% of total oxygen consumption is estimated to be reduced to superoxide (45). The concentration of superoxide is kept remarkably low by superoxide dismutase. Although the brain is commonly assumed to be poorly defended against oxidants, ~0.5% of total soluble protein in brain is copper, zinc superoxide dismutase (29). The intracellular concentration of superoxide dismutase is ~4–10 μM in both brain and liver. Thus tissues have a formidable drain to remove superoxide.

In effect, peroxynitrite is a binary toxin assembled mainly when cells make large amounts of both nitric oxide and superoxide (Fig. 7). Because eukaryotic cells contain large amounts of superoxide dismutase, the critical control becomes whether the concentration of nitric oxide approaches that of superoxide dismutase. Inducible nitric oxide synthase can make substantial

$$O_2^{\cdot-} \nearrow \text{ONOO}^- \quad 6.7 \times 10^9 \ \text{M}^{-1}\cdot\text{s}^{-1} \times [\text{NO}]$$

$$O_2^{\cdot-} \searrow O_2 + H_2O_2 \quad 2 \times 10^9 \ \text{M}^{-1}\cdot\text{s}^{-1} \times [\text{SOD}]$$
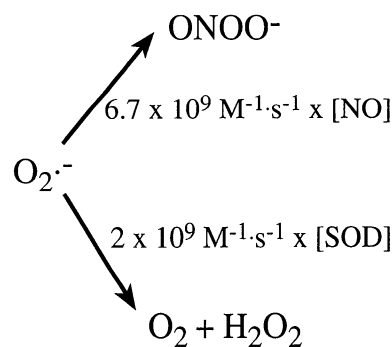
Fig. 7. Fate of superoxide is controlled by its reaction rates with possible targets multiplied by the target concentration. Superoxide dismutase is present in micromolar concentrations and reacts at $2 \times 10^9 \ \text{M}^{-1}\cdot\text{s}^{-1}$ and is generally the major drain for removing superoxide. Concentrations of nitric oxide that cause vasorelaxation are on the order of 5–10 nM and will not effectively compete with superoxide dismutase for superoxide. When the concentration of nitric oxide rises to micromolar concentrations, it can effectively compete with superoxide dismutase because of its rapid rate constant.

concentrations of nitric oxide when expressed. However, neuronal nitric oxide synthase could potentially make enough if all cells in one region are fully activated. For example, Malinski et al. (61) have measured 2–4 µM nitric oxide in ischemic brain during cerebral ischemia, under conditions when the inducible nitric oxide synthase should not have been present.

What makes peroxynitrite particularly toxic is its remarkable stability as an anion at alkaline pH. Molar concentrations at pH 13 can be stored for days in the refrigerator, and a pure solid form has recently been prepared (9). The stability of peroxynitrite allows a greater opportunity for it to diffuse through a cell to find a target. The unusual stability of peroxynitrite is due to its being folded into the *cis*-conformation (Fig. 8), which cannot directly isomerize to the much more stable form, nitrate (96). The apparent pK_a of *cis*-peroxynitrite is 6.8 (53), so that at pH 7.4 only 20% of peroxynitrite will be protonated to form peroxynitrous acid. When protonated, peroxynitrite can isomerize to the *trans*-conformation, which appears to be more reactive and is also capable of rearranging to nitrate (18).

Peroxynitrous acid is a strong oxidant that can react with biological molecules by a number of complex mechanisms. It is particularly efficient at oxidizing iron/sulfur centers (12, 39), zinc fingers (17), and protein thiols (80). It can also produce the products expected for hydroxyl radical attack (3, 18). Thus peroxynitrite leaves the hallmarks of oxidation traditionally used to implicate free radicals in vivo, including formation of protein carbonyls, salicylate, and 8-hydroxyguanidine, and the formation of HO· adducts on spin traps (1). However, the direct reaction of peroxynitrite with specific moieties is more likely to account for its toxicity (53). For example, incubation of *Escherichia coli* with hydroxyl radical scavengers did not reduce the cytotoxicity of peroxynitrite (109) and, in the case of dimethyl sulfoxide, increased toxicity. Peroxynitrite is toxic by

Fig. 9. Structure of nitrotyrosine and nitrosotyrosine. The nitroso group can apparently interchange between the carbon and oxygen. Nitrosotyrosine is unstable in water, because water can react with the NO group to form nitrite and tyrosine. Nitrotyrosine is stable in proteins in vitro for many years.

more direct oxidative mechanisms. The underlying chemistry of peroxynitrite is sufficiently complex that it will continue to entertain inorganic chemists and quantum mechanical theorists for many years (79).

One important reaction of peroxynitrite is catalyzed by transition metals, including the metal centers of superoxide dismutase and myeloperoxidase (47, 86). Transition metals catalyze a heterolytic cleavage to produce hydroxyl anion plus nitronium ion (NO_2^+). Nitronium ion is well known to attack phenolics to produce nitrophenols, but it is usually formed with concentrated mixtures of nitric and sulfuric acid. The nitration of protein tyrosine residues to give 3-nitrotyrosine is a footprint left by peroxynitrite in vivo (Fig. 9). This reaction is catalyzed by superoxide dismutase, the enzyme that is also responsible for minimizing the formation of peroxynitrite by scavenging superoxide (6).

The reaction of superoxide dismutase with peroxynitrite can be a useful assay for peroxynitrite in vivo, even though it traps only 8–9% of the available peroxynitrite. However, superoxide dismutase can also inhibit the formation of peroxynitrite by directly scavenging superoxide before it reacts with superoxide dismutase. Copper, zinc superoxide dismutase can be chemically modified to circumvent this competing reaction. Greater than 99% of the superoxide scavenging activity of superoxide dismutase can be inactivated by chemical modification, without significantly affecting the reaction with peroxynitrite (47).

We utilized the nitration of the low-molecular-weight phenolic 4-hydroxyphenylacetic acid catalyzed by superoxide dismutase as a trap to assay peroxynitrite formation by activated rat alveolar macrophages (46). Freshly isolated rat alveolar macrophages were able to produce nitric oxide without stimulation but did not nitrate phenolics. Phorbol esters stimulated superoxide production at a fourfold greater rate than nitric oxide production. In the presence of native superoxide dismutase or the chemically modified superoxide dismutase, the solution turned slightly yellow as the phenolic was nitrated. Quantitatively, all of the nitric oxide produced by macrophages under these conditions was converted to peroxynitrite, even with 5 µM extracellular superoxide dismutase added.

We have recently raised both polyclonal and monoclonal antibodies to nitrotyrosine-containing proteins.
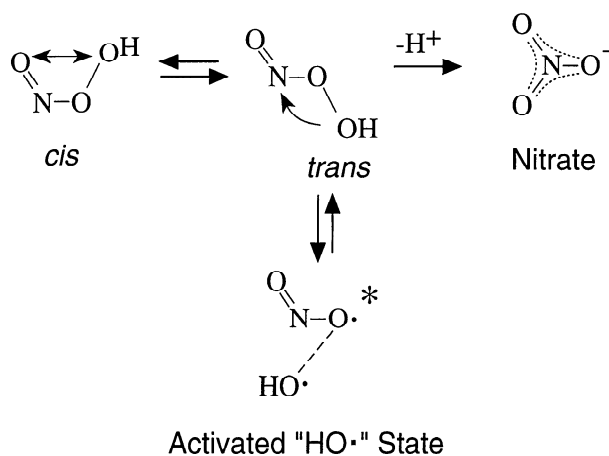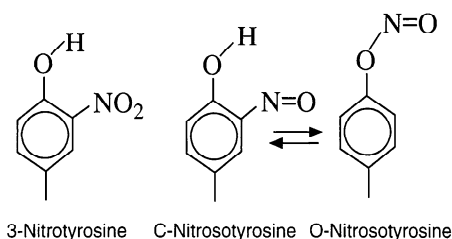
Activated "HO·" State

Fig. 8. Peroxynitrite is most stable when folded into a *cis*-conformation. When protonated, it will isomerize to *trans*-peroxynitrous acid. *Trans*-peroxynitrous acid can form an excited state that can react like hydroxyl radical plus nitrogen dioxide. The distal peroxy oxygen in *trans*-peroxynitrous acid can also swing around to attack the nitrogen to form nitrate.

These can be used for both immunohistochemistry and Western blotting (7). We have shown that extensive nitration takes place around foamy macrophages in human atherosclerotic lesions (7). Additional staining is found in foci of myocytes in the vascular smooth muscle. Staining of foamy macrophages was also observed in early fatty streaks.

A major concern with any antibody is potential nonspecificity. Antibody binding can be blocked by either millimolar concentrations of nitrotyrosine or micromolar concentrations of short peptides containing nitrotyrosine. Thus the antibodies are selective for nitrotyrosine incorporated into proteins. Binding is not affected by tyrosine, chlorotyrosine, phosphotyrosine, or aminotyrosine. Specificity can be further investigated by treating the samples with dithionite, which will chemically reduce nitrotyrosine to aminotyrosine. The combination of blocking and reducing agents provides excellent controls that can be used even on human autopsy tissue to verify the specificity of nitrotyrosine binding.

Tyrosine nitration is found in human lung biopsy and autopsy samples with sepsis, pneumonia, or adult respiratory distress syndrome (38, 52). Control lung samples show relatively little nitration. Exposure of rats to 100% oxygen will also induce nitration in lung alveoli at the time when pathology becomes apparent. Myocytes in human heart from patients with system sepsis or myocarditis show extensive nitration. By Western blot analysis, actin appears to be nitrated in heart (7). Myocardial depression associated with nitric oxide production (97) may be due to nitration of the contractile protein machinery of muscle cells. Experimentally, actin has been artificially nitrated with tetranitromethane and was shown to assemble abnormally (13, 65).

Tyrosine nitration is a convenient marker of reactive nitrogen-centered oxidants being produced. It is not necessarily due to the formation of peroxynitrite, but peroxynitrite is the most likely source in vivo (7). Other reactive species like nitrogen dioxide can form nitrotyrosine in simple solutions. Acidified nitrite can also produce nitrotyrosine if left for several days in contact with a protein. However, the amounts of nitrogen dioxide or nitrite that are present in vivo are far lower than necessary to cause significant nitration in vitro. If small amounts of ascorbate or thiols are present, they will strongly inhibit tyrosine nitration by nitrogen dioxide (77). In a complex media such as the milieu of a cell, the reaction of superoxide dismutase with peroxynitrite becomes important because the superoxide dismutase combines rapidly with peroxynitrite and directs a more selective nitration of tyrosines on certain proteins. The vast number of alternative targets in biological materials for peroxynitrite makes the superoxide dismutase-peroxynitrite complex more selective.

We have recently found that nitration of neurofilament L is greatly enhanced by superoxide dismutase. This may contribute to the role of superoxide dismutase in causing amyotrophic lateral sclerosis (ALS). Over 35 different dominant missense mutations at 26 different amino acid positions in superoxide dismutase have been associated with the familial form of ALS (21, 83). Expression of these mutations in transgenic mice causes development of motor neuron disease, whereas equivalent expression of the wild-type enzyme does not (37). These results strongly suggest that an enhanced deleterious function of superoxide dismutase is responsible for the demise of motor neurons, yet superoxide dismutase is expressed in high concentrations in all cells, which makes the selective vulnerability of motor neurons difficult to explain.

We have proposed that the deleterious function is enhanced nitration of a key tyrosine kinase target (4). However, superoxide dismutase-catalyzed nitration of neurofilaments may be a more likely explanation (Fig. 10). The head domain of human neurofilament L contains 10 tyrosines in the first 96 amino acids. Neurofilaments are intermediate filaments that provide structural stability and control diameter in motor neurons (10). Motor neurons are the largest neurons in the central nervous system and contain more neurofilaments than other cell types. Recently, the overexpression or mutation of neurofilaments has been shown to

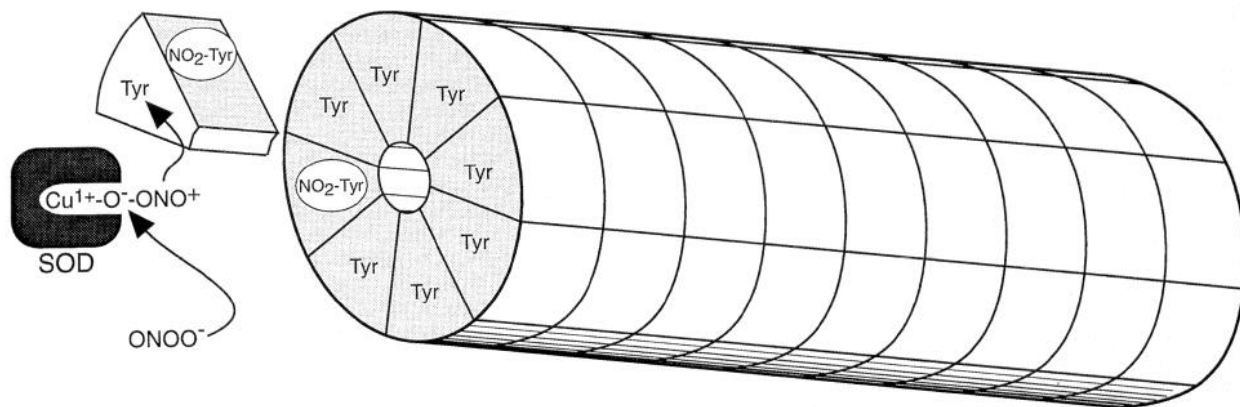
Fig. 10. Structural proteins appear to be major targets for superoxide dismutase-catalyzed nitration by peroxynitrite. When disassembled, many of the tyrosines involved in intersubunit contacts are exposed to solvent and susceptible to nitration. Because nitrotyrosine is negatively charged, incorporation of just one modified subunit could potentially disrupt the assembly of an entire filament.

cause severe motor neuron injury in transgenic mice (57, 107). We have found that superoxide dismutase greatly enhances the nitration of neurofilaments in vitro and have detected nitrated neurofilament L in autopsy tissue from ALS spinal cord patients (J. P. Crow and M. Strong, unpublished observations). Nitration of tyrosines will change a normally hydrophobic residue into a negatively charged hydrophilic residue and thereby disrupt the assembly of these proteins into a long polymeric structure.

Nitration of cytoskeletal proteins is likely to occur in other diseases because structural proteins are abundant and relatively stable. Many of the tyrosines involved in intersubunit contacts are exposed to solvent as unassembled subunits, which makes the tyrosines accessible to peroxynitrite-bound superoxide dismutase (Fig. 10). Because nitrotyrosine can be negatively charged at neutral pH, nitration can seriously compromise the ability of subunits to correctly assemble into filaments.

In summary, the biological chemistry of nitric oxide can be largely summarized by three principal reactions: binding to ferrous heme in guanylate cyclase to activate cGMP, elimination by oxyhemoglobin, and formation of toxic oxidants by reacting with superoxide. These three reactions are certainly a simplification but a reasonable summary because they are rapid under physiologically relevant conditions. Diffusion is essential for understanding the ability of nitric oxide to act as a local modulator and is a major determinant of biological lifetime.

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