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NOVEL EFFECTS OF NITRIC OXIDE

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■ **Abstract** Nitric oxide (NO), a simple free radical gas, elicits a surprisingly wide range of physiological and pathophysiological effects. NO interacts with soluble guanylate cyclase to evoke many of these effects. However, NO can also interact with molecular oxygen and superoxide radicals to produce reactive nitrogen species that can modify a number of macromolecules including proteins, lipids, and nucleic acids. NO can also interact directly with transition metals. Here, we have reviewed the non–3′,5′-cyclic-guanosine-monophosphate–mediated effects of NO including modifications of proteins, lipids, and nucleic acids.

INTRODUCTION

Nitric oxide (NO) has been implicated in a diverse collection of physiological functions including smooth muscle relaxation, inhibition of platelet activation, neurotransmission, and immune response (1, 2). NO is formed from L-arginine by the enzyme nitric oxide synthase (NOS). In addition to the L-arginine substrate, the reaction catalyzed by NOS requires molecular oxygen, NADPH, and other cofactors such as tetrahydrobiopterin, FMN, FAD, and heme to produce NO and citrulline (3).

The three isoforms of NOS are encoded by distinct genes (1, 3). NOS-1, also known as neuronal or brain NOS (nNOS) or Type I NOS, is found in high concentrations in neuronal and some nonneuronal tissues (1, 3). NOS-2 is also known as Type II NOS, macrophage NOS, or inducible NOS (iNOS). Although this isoform of NOS was originally found in macrophages, it exists in a variety of cell types including hepatocytes, vascular smooth muscle cells, fibroblasts, and epithelial cells (1, 3). NOS-3, or endothelial NOS (eNOS) or Type III NOS, was first identified as the enzyme that produces endothelium-derived relaxing factor (1, 3). Both NOS-1 and NOS-3, often grouped together as constitutive NOS (cNOS), are usually constitutively expressed, and their activities are regulated by intracellular calcium

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concentrations via calmodulin. NOS-2, on the other hand, is not expressed in resting cells. Compounds, such as endotoxin or proinflammatory cytokines (including interleukin-1, IFNγ, and tumor necrosis factor alpha), induce iNOS expression (1, 3). NOS-2 is bound to calmodulin even under resting intracellular calcium concentrations; thus, the function of NOS-2 is not affected by intracellular calcium concentrations. In addition, NOS-2 can produce much higher levels of NO compared with NOS-1 or NOS-3. These higher NO levels are thought to be responsible for the bacterial cytotoxic characteristics of cells expressing the NOS-2 isoform (1, 3).

In addition to its physiological functions, NO has also been implicated in the pathology of many inflammatory diseases, including arthritis, myocarditis, colitis, and nephritis, and a large number of pathological conditions such as amyotrophic lateral sclerosis (ALS), cancer, diabetes, and neurodegenerative diseases (4–15). Although NO has been the subject of much research and a huge number of publications, the role NO plays in many of these diseases is controversial. NO often has opposing effects, depending upon the experimental model, the relative concentration of NO, and the surrounding milieu in which NO is produced, resulting in much confusion in the literature. There are both direct effects of NO, mediated by the NO molecule itself, and indirect effects of NO, mediated by reactive nitrogen species (RNS) produced by the interaction of NO with oxygen (O_2) or superoxide radicals ($O2^{\bullet -}$). 3′,5′-cyclic guanosine monophosphate (cGMP), produced by the interaction of NO with soluble guanylate cyclase, mediates many of the direct effects of NO. As the effects of NO are numerous, and cGMP-mediated NO actions have been discussed thoroughly in other reviews, we discuss only the non-cGMP-mediated effects of NO.

CHEMISTRY OF NITRIC OXIDE

To understand the pathophysiological effects of NO and the paradoxical effects that NO sometimes exhibits, one must first understand the biological chemistry of NO. The chemistry of NO, which we discuss only briefly here, is described very succinctly and thoroughly in several recent reviews (16, 17). Figure 1 summarizes the chemistry of NO. When discussing the chemistry and physiological effects of NO, it should be remembered that NO is a highly diffusible second messenger that can elicit effects relatively far from its site of production. The concentration, and therefore the source of NO, are the major factors determining the biological effect of NO (16). At low concentrations ($<1\ \mu$M), the direct effects of NO predominate. At higher concentrations ($>1\ \mu$M), the indirect effects mediated by RNS prevail. Thus, in cell types that contain cNOS isoforms NO is produced in relatively low amounts for short periods of time and elicits mostly direct effects. When iNOS is induced, high concentrations of NO are produced, and the indirect and often pathologic effects of NO are exhibited.

The direct effects of NO most often involve the interaction of NO with metal complexes. The reactions of NO with heme-containing proteins are the most

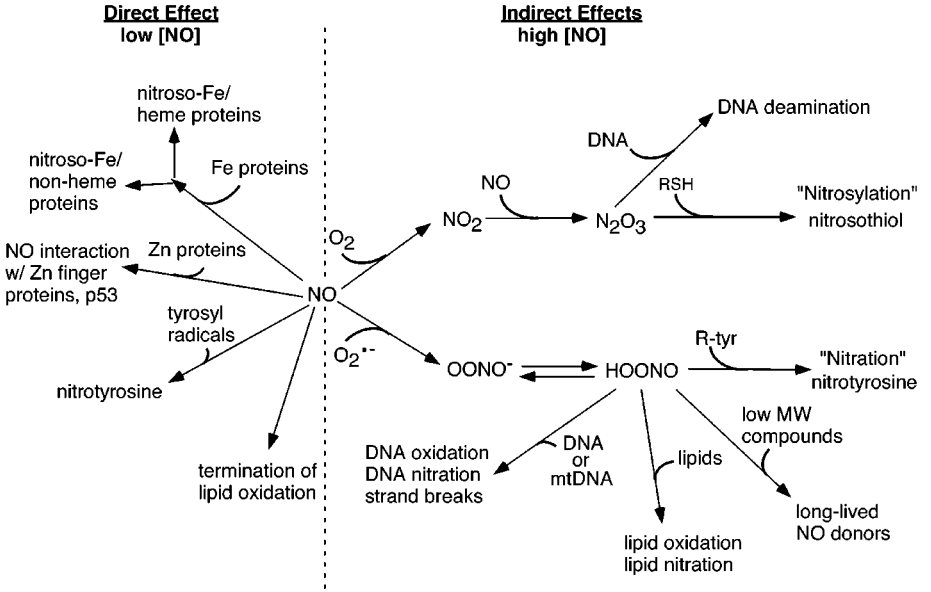

Figure 1 Summary of the chemistry of NO. The direct effects of NO, shown on the left, are usually elicited at low NO concentrations, whereas the indirect effects of NO, shown on the right side, are elicited at high NO concentrations.

physiologically relevant and include interactions with soluble guanylate cyclase (18) and cytochrome P450 (19). However, NO can also interact with nonheme iron-containing proteins and zinc-containing proteins.

The indirect effects of NO, produced through the interaction of NO with either O_2 or $O_2^{\bullet -}$, include nitrosation (when NO+ is added to an amine, thiol, or hydroxy aromatic group), oxidation (when one or two electrons are removed from a substrate), or nitration (when NO_2^+ is added to a molecule) (16). In aqueous solutions NO can undergo autoxidation (i.e. reaction with O_2) to produce N_2O_3; this compound can undergo hydrolysis to form nitrite (20, 20a). The autoxidation rate is second order with respect to NO; thus, the half-life of NO depends on its concentration (20). This brings into focus the importance of temporal and spatial factors when considering the effects of NO. NO diffuses easily from its site of production. As distance from the site of production increases, the concentration of NO decreases and the half-life increases. At lower concentrations, farther away from the site of NO production, the direct effects of NO predominate. However, closer to the site of production of large amounts of NO, the indirect effects of NO predominate.

The rate constant for the autoxidation of NO is unaffected by pH, temperature, or hydrophobicity (16, 20). Thus, the rate of autoxidation is dependent only on NO and O_2 concentrations. Because NO and O_2 are 6–20 times more soluble in lipid layers compared with aqueous fractions, the rate of autoxidation is increased dramatically

in the lipid phase (20, 20b, 20c). Thus, the primary reactions of N_2O_3, nitrosation of thiols and amines, are thought to occur primarily in the membrane fraction.

In addition to autoxidation, NO also reacts with $O2^{\bullet-}$ to produce the powerful oxidant, peroxynitrite ($ONOO^-$) (21). This reaction occurs at near diffusion rates ($7 \times 10^9\ M^{-1}\,s^{-1}$). $ONOO^-$ acts as both a nitrating agent and a powerful oxidant to modify proteins, lipids, and nucleic acids. However, several factors limit the rate of production and the physiological effects of $ONOO^-$. The availability of NO and $O2^{\bullet-}$ are critical. Superoxide dismutase (SOD) scavenges $O_2{}^{\bullet-}$ at a rate of $2 \times 10^9\ M^{-1}\,s^{-1}$, close to the rate of $ONOO^-$ formation. Thus, when SOD is present in micromolar concentrations, it can compete effectively for $O_2{}^{\bullet-}$. In a similar fashion, NO reacts rapidly with heme proteins, especially oxyhemoglobin, limiting NO diffusion in vivo to the site of $O2^{\bullet-}$ production.

The relative amounts of NO and $O_2{}^{\bullet-}$ are also critical in the production of $ONOO^-$. $ONOO^-$ can react with both NO and $O_2{}^{\bullet-}$ to form nitrogen dioxide (NO_2) (16). Thus, an overproduction of either NO or $O_2{}^{\bullet-}$ will decrease the reactivity of $ONOO^-$; accordingly, the maximum activity of $ONOO^-$ occurs when $O_2{}^{\bullet-}$ and NO are produced in equivalent amounts. When there is more production of NO than $O_2{}^{\bullet-}$, NO_2 is produced, which can then react with NO to produce the nitrosating agent N_2O_3. When there is an overproduction of $O_2{}^{\bullet-}$, oxidative chemistry prevails.

There are two likely sources of $O_2{}^{\bullet-}$: mitochondria and immune cells. Mitochondria produce $O_2{}^{\bullet-}$ during the course of aerobic respiration. As NO is more soluble in lipid layers, $ONOO^-$ may be formed in the hydrophobic regions of mitochondria. However, the mitochondrial manganese SOD (MnSOD) may play a role in limiting the production of $ONOO^-$ under normal conditions. Under inflammatory conditions immune cells such as neutrophils and macrophages produce large quantities of $O_2{}^{\bullet-}$ through either NADPH oxidase or xanthine oxidase (22). Macrophages also produce large quantities of NO; thus, $ONOO^-$ is likely to be produced under inflammatory conditions. Immune cells may also produce nitrating species through the myeloperoxidase system.

In summary, the potential reactions of NO are numerous and dependent on many different factors. The site and source of production, as well as the concentration of NO, collectively determine whether NO will elicit direct or indirect effects. In addition, a relative balance between oxidative and nitrosative stress exists that will determine the indirect effects of NO.

NO MODIFICATIONS OF PROTEINS

S-Nitrosylation

S-nitrosylation of cysteine residues resulting from the addition of a NO+ group has been shown to modify the activity of several proteins. Although it is unlikely that NO acts directly on the cysteine residue, NO interacts with O_2 or $O_2{}^{\bullet-}$ to produce

RNS capable of nitrosylating cysteine residues. Nitrosylation is a chemical reaction, not an enzymatically catalyzed reaction. However, there seems to be some specificity in nitrosylation. First , not every protein with available cysteine residues becomes nitrosylated. Subcellular location and the local chemical environment (i.e. the local concentration of NO and molecules that react with NO such as $O_2^{\bullet -}$ and heme proteins) may dictate to some extent which proteins become nitrosylated. Furthermore, not every available cysteine residue within a given target protein becomes nitrosylated. Of the five cysteine residues in $p21^{Ras}$, only one cysteine is nitrosylated (23). In the ryanodine receptor there are 84 cysteines with free $-$SH groups, but only 12 cysteines appear to be susceptible to nitrosylation (24). The tertiary structure of a protein may make some cysteine residues more susceptible to nitrosylation. A consensus sequence, though somewhat degenerate, for nitrosylation has also been postulated. The proposed motif is XYCZ, where X is Gly, Ser, Thr, Cys, Tyr, Asn, or Gln; Y is Lys, Arg, His, Asp or Glu; and Z is Asp or Glu (25).

Nitrosylation has been shown to modify the function of several proteins including the N-methyl-D-aspartate (NMDA) receptor, $p21^{Ras}$, caspase-3, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which suggests that nitrosylation may be an important cellular regulatory mechanism. The NMDA receptor is a glutamate receptor critical for development, learning, and memory in the central nervous system (26). Activation of the NMDA receptor triggers Ca^{2+} influx, which causes nNOS activation and subsequent NO production (27, 28). Manzoni & Bockaert showed that NMDA receptor activity is downregulated by endogenous NO in primary neurons (29). This downregulation was later shown, through site directed mutagenesis experiments, to be due to specific nitrosylation of cysteine 399 in the NR2A subunit of the NMDA receptor (30). Downregulation of NMDA receptor function by NO may act as a negative feedback mechanism to prevent excessive activation of the NMDA receptor and associated neurotoxicity (31).

Although NO has been shown to be neuroprotective, it has also been shown to be neurotoxic in some circumstances. The paradoxical effects of NO in neurons are an example in which the effects of NO are dictated by the intracellular milieu. When NO is produced under conditions in which $O_2^{\bullet -}$ is available, NO has neurotoxic effects in rat cerobrocortical cultures (a mixture of neuronal and glial cells) (32). When cells are treated with SOD, NO does not produce these toxic effects; however, exogenous treatment of the cerobrocortical cultures with peroxynitrite and SOD causes neurotoxicity (32). Furthermore, treatment of neurons with NO congeners in different redox states resulted in different effects. 3-morpholinosydnonimime (SIN-1), which produces both NO. and $O_2^{\bullet -}$, caused neurotoxicity; nitroglycerin and sodium nitroprusside, which both produce NO+ equivalents, cause neuroprotective effects (32). The neurotoxic effects of NO seem to be related to the formation of peroxynitrite; however, it is unclear whether the deleterious effects of peroxynitrite are on the NMDA receptor or elsewhere in the cell. It is clear that when cellular conditions favor nitrosylation, NO has neuroprotective effects; however, if NO is produced under conditions in which there is also $O_2^{\bullet -}$ production, NO can be neurotoxic.

In addition to the NMDA receptor, nitrosylation has also been shown to modulate the activity of several other channels. Nitrosylation of a single cysteine residue on the intracellular face of the cyclic nucleotide-gated channel involved in olfactory and visual transduction results in activation of this channel (33, 34). Polynitrosylation of the cardiac calcium release channel (ryanodine receptor) also results in activation of this channel (24). In this case, nitrosylation of up to 12 cysteine residues of the available 84 leads to progressive, reversible activation of the channel (24).

Recently, NO has been shown to modulate cell survival by interacting with several proteins in proapoptotic pathways. One such protein whose function is modified by nitrosylation is $p21^{ras}$. Lander and coworkers showed that NO activates $p21^{ras}$ in human T cells (23). Furthermore, in vitro experiments showed that this activation is a direct, reversible effect mediated by NO (23). The concentration of NO necessary to elicit maximal activation in vitro was more than 1000 times higher compared to that required in whole cells, which suggests that intracellular conditions are more favorable for nitrosothiol formation. Lander subsequently showed that $p21^{ras}$ is specifically nitrosylated on cysteine 118 (35). When cysteine 118 is mutated to a serine (C118S mutant), NO mediated activation of p21ras is eliminated (35).

In PC12 pheochromocytoma cells, $p21^{ras}$ mediates neuronal growth factor-driven differentiation and survival through the recruitment of the mSOS-Ras-MAP kinase cascade (mSOS-Ras-Raf-MEK-ERK) and phosphatidylinositol (PI) 3–kinase, respectively (36–39). The mSOS-Ras-MAP kinase pathway is normal in the C118S- expressing PC12 cells compared to wild type Ras-expressing PC12 cells. However, after long-term neuronal growth factor treatment, C118S-expressing PC12 cells are unable to maintain PI 3-kinase activation resulting in apoptosis of these cells (40). Thus, while nitrosylation of Ras does not affect the mSOS-Ras-MAP kinase pathway, it does modulate the interaction of Ras with the PI 3–kinase pathway. These experiments demonstrate that NO, acting as an anti-apoptotic agent, activates $p21^{ras}$ to modulate neuronal PC12 survival but not differentiation (40).

NO has also been shown to nitrosylate several members of the caspase family of proteins, which functions in the apoptotic pathway. However, in this case nitrosylation results in inhibition of activity instead of activation, as observed with $p21^{ras}$. NO has been shown (in vitro) to inhibit seven members of the caspase family, including caspases-1, -2, -3, -4, -6, -7, and -8, through mitrosylation (41). Caspases contain a reactive cysteine residue in the active site of the enzyme, which is specifically nitrosylated by NO donors (42). Nitrosylation of caspase appears to be reversible, probably depending on the redox state of the cell, which suggests that NO-mediated inhibition of apoptosis is reversible (43).

Caspase-1 is part of the caspase subfamily that participates in cytokine maturation. In human umbilical vein endothelial cells (HUVEC), NO was shown to inhibit tumor necrosis factor (TNF)-α-induced apoptosis through s-nitrosylation of caspase-1 (44). Both exogenous treatment of cells with NO donors and endogenous

activation of NOS by shear stress antagonized TNF-α-induced apoptosis in a cGMP-independent manner. Interestingly, whereas low concentrations of NO donors ($<$50 uM sodium nitroprusside or S-nitrosopenicillamine) were protective through the inhibition of caspase-1 activity, higher concentrations of NO ($>$300 uM) were pro-apoptotic, revealing once again the paradoxic effects of NO (44).

Treatment of purified caspase-1 with NO resulted in nitrosylation of the active site cysteine residue and inhibition of caspase activity (44). Site-directed mutagenesis experiments showed that caspase-3 is also nitrosylated on its active site cysteine (45). Furthermore, endogenous caspase-3 nitrosylation has been documented. Experiments in human B and T cell lines have demonstrated a biological role for caspase nitrosylation in apoptosis. A significant portion of caspase-3 was found to be nitrosylated in human B and T cell lines (including 10C9 and Jurkat cells) inhibiting caspase activity (45). Activation of Fas-dependent apoptosis in these cells causes a denitrosylation of caspase-3 within 1.5–2.0 hours (45). Incubation of Jurkat and 10C9 cells with NOS inhibitors for long periods of time (24 hours) resulted in denitrosylation of caspase-3 but not activation. However, the denitrosylation caused an increase in Fas-induced activation of caspase-3. Thus, both denitrosylation and cleavage seem to be required for caspase activation. When the Fas apoptotic pathway is induced, caspase-3 is denitrosylated to expose the active site cysteine so that, upon Fas-induced cleavage, caspase-3 becomes active. In this way, NO regulates the Fas apoptotic pathway through the balance of nitrosylation/denitrosylation (45, 46).

GAPDH was among the first proteins shown to be nitrosylated. A correlation was found between NO production and inhibition of GAPDH in rat liver during chronic inflammation (47). GAPDH contains a cysteine residue in its active site that is thought to be nitrosylated, leading to reversible inhibition of GAPDH activity (47). Moreover, the S-nitrosylation of GAPDH facilitates further covalent modification of the enzyme by NADH (48). Nitrosylation of GAPDH is reversible and may be involved in the regulation of glycolysis; however, NADH modification of GAPDH is irreversible and is likely to be involved in pathological events (48). Several other enzymes, including aldolase, aldehyde dehydrogenase, cathepsin B, and γ-glutamylcysteinyl synthetase, are nitrosylated on their active site cysteine residues, in a manner similar to GAPDH, and are reversibly inactivated(49).

Tyrosine Nitration

Nitrotyrosine formation has been demonstrated by immunohistological staining in numerous human diseases and animal models. Nitration of tyrosine residues is thought to be selective. Western blotting of nitrotyrosine-containing tissues in our laboratory revealed that only a few proteins appear to be nitrated in vivo. In skeletal muscle, the SERCA2a isoform of the sarcoplasmic reticulum Ca-ATPase is nitrated. In in vitro experiments SERCA2A is preferentially nitrated, even when the SERCA1 isoform is added in excess of SERCA2A (50). It is worth noting that

although tyrosine nitration has been demonstrated in many pathological conditions, nitrotyrosine formation also seems to occur under basal conditions.

In addition to selectivity, there is increasing evidence that tyrosine nitration is reversible. In a paper recently published in *Proceedings of the National Academy of Sciences*, our laboratory described an activity in crude rat spleen and lung homogenates that removes the nitrotyrosine epitope of nitrated proteins (51). It is unclear what type of modification this factor is making to the nitrotyrosine epitope (the product of the reaction is currently being determined). We have found this activity not only in spleen but also in several other tissues. Other investigators have also shown evidence that tyrosine nitration is reversible. When Gow et al incubated nitrotyrosine-containing peptides with plasma, the amount of nitrated peptides was reduced, which suggests that tyrosine nitration may be reversible (52).

The in vivo pathway of tyrosine nitration has been a source of controversy for several years. Figure 2 shows the potential pathways for tyrosine nitration. Peroxynitrite, formed from the reaction of NO with $O_2^{\bullet -}$, was thought to be the major nitrating agent in vivo. As a matter of fact, nitrotyrosine is routinely used as a marker for peroxynitrite production. However, there may be peroxynitrite-independent mechanisms of tyrosine nitration; thus, nitrotyrosine may not be a reliable marker for peroxynitrite production. There are a number of in vitro chemical studies showing that peroxynitrite can nitrate tyrosine residues (53, 54). Furthermore, peroxynitrite has been shown to nitrate tyrosine residues in intact cells (55). In contrast to these studies, Pfeiffer and Mayer observed very little tyrosine nitration at physiologic pH when $NO^{\bullet}$ (spermine NONOate) and $O_2^{\bullet -}$ (xanthine oxidase) were generated simultaneously to form peroxynitrite compared to treatment with preformed peroxynitrite causing these authors to suggest that peroxynitrite may not be a nitrating agent in vivo (56). However, several investigators have questioned Pfeiffer and Mayer's studies in two recently published papers where efficient tyrosine nitration by the simultaneous production of $NO^{\bullet}$ and $O_2^{\bullet -}$was demonstrated (56a, 56b). Sawa and Reiter show that the accumulation of urate and the rapid consumption of oxygen by xanthine oxidase in the Pfeiffer and Mayer study may have led to erroneous conclusions (56a, 56b).

An alternative pathway for peroxynitrite-mediated tyrosine nitration is the interaction of peroxynitrite with CO_2 (rate constant, 5.8×10^4 M^{-1} s^{-1}) to form nitrating species. Squadrito & Pryor proposed that the reaction of peroxynitrite with CO_2 yields the free radicals $NO^{\bullet}{}_2$ and $CO_3^{\bullet -}$, which can nitrate phenolic compounds such as tyrosine (57).

Recent evidence suggests there may be peroxynitrite-independent mechanisms by which nitrotyrosine can form in vivo. Myeloperoxidase (MPO) uses H_2O_2 and Cl^- to produce HOCl. Eiserich proposed that NO_2^- formed from the oxidation of NO can be oxidized by either HOCl or myeloperoxidase to form reactive nitrogen species, NO_2Cl and NO_2, which may be capable of nitrating tyrosine residues (58, 59). Eiserich et al used human polymorphonuclear neutrophils to show that the MPO system can generate RNS capable of nitrating the tyrosine residues

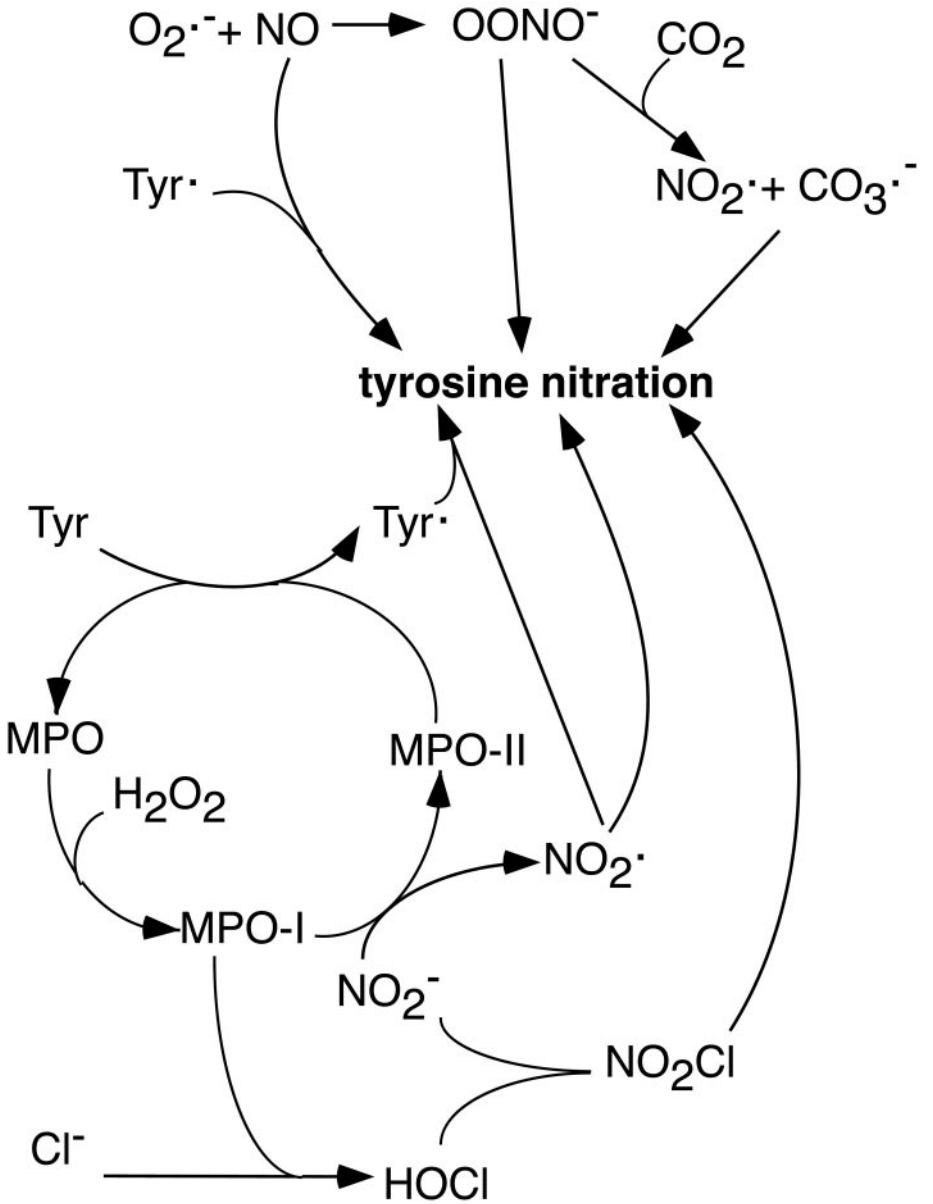

Figure 2 The proposed mechanisms for in vivo nitration of tyrosine residues. The MPO-mediated mechanisms are demonstrated on the bottom, and peroxynitrite and tyrosyl radical mechanisms are shown at the top. MPO, myeloperoxidase; tyr●, tyrosyl radical.

of several synthetic peptides (58). There is also recent evidence that eosinophil peroxidase may catalyze the production of nitrating species (60).

In contrast to Eiserich's proposed mechanism, van Dalen et al showed that NO_2^- is a poor substrate for MPO (61). Thus, in the presence of physiological levels of NO_2^- and Cl^-, MPO catalyzes very little tyrosine nitration. However, van Dalen proposed an alternative mechanism for MPO-mediated tyrosine nitration (61). MPO may catalyze the oxidation of free tyrosyl residues to tyrosyl radicals that can then exchange for tyrosyl residues in proteins. These tyrosyl radicals can then be nitrated by NO_2. Thus, free tyrosine acts as a cosubstrate in MPO-mediated tyrosine nitration.

Another pathway for the formation of nitrotyrosine is through the direct reaction of NO with a tyrosyl radical. NO can form an unstable complex with the tyrosyl residue of prostaglandin H synthase-2 (62). This complex can be oxidized to form a nitrotyrosine (62). This pathway necessitates the presence of a tyrosyl radical in the protein and thus, may be of limited consequence in biological systems. However, there are a number of proteins that contain tyrosyl radicals and may undergo similar chemistry, namely ribonucleotide reductase and photosystem II (63, 64).

Nitrotyrosine formation has gained increasing attention. The observation of tyrosine nitration in a number of human diseases has brought the idea that nitration of tyrosine residues in proteins has functional consequences to the forefront of NO research in recent years. Nitrotyrosine has been detected in atherosclerotic plaques of coronaries by both immunohistochemistry and Western blotting (65, 66). Increased nitrotyrosine staining was found in motor neurons of patients with ALS (9). Tyrosine nitration has also been found in rejected renal allografts and chronic renal failure, inflammatory bowel disease, the synovial fluid of arthritis patients, and the placental tissues from preeclamptic pregnancies (67–71).

Nitrotyrosine formation is also found in numerous animal models of disease. In the heart, nitrotyrosine formation has been detected in ischemia-reperfusion injury and myocardial inflammation (72, 73). Tyrosine nitration has also been found in the kidney in endotoxin-induced injury and in renal hypertension (74, 75). Nitration is evident in an MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-induced model of Parkinson's disease and in a transgenic model of ALS (76, 77). The list of pathological conditions in which nitrotyrosine formation is observed is extensive; however, in very few of these diseases have the modified protein(s) been identified. Furthermore, many proteins that have been shown to be nitrated by peroxynitrite in vitro have never been conclusively shown to be nitrated in vivo. Thus, the physiological consequences of tyrosine nitration remain poorly understood.

One protein that has been shown to be nitrated in vivo is prostacyclin synthase. Nitration of prostacyclin synthase has been demonstrated in both interleukin 1β (IL-1β)–stimulated rat mesangial cells and in atherosclerotic bovine coronary arteries (78, 79). In rat mesangial cells prostacyclin synthase was immunoprecipitated from IL-1β–treated cells using antinitrotyrosine antibodies. Decreased prostacyclin synthase activity in these cells as well as decreased activity of prostacyclin synthase, treated in vitro with peroxynitrite, indicates that nitration

causes inhibition of prostacyclin synthase (78, 80). In bovine coronary arteries immunoprecipitations using prostacyclin synthase antibodies revealed increased staining for nitrotyrosine in atherosclerotic arteries compared with normal tissue. Vasodilation was impaired in the atherosclerotic vessels compared with normal vessels, which suggests that inhibition of prostacyclin synthase by nitration was at least partially responsible for impaired relaxation in these vessels.

Manganese superoxide dismutase (Mn SOD) was also found to be nitrated in vivo. Mn SOD was extracted from human renal allografts by immunoprecipitation using antinitrotyrosine antibodies (67). More Mn SOD was immunoprecipitated from chronically rejected allografts compared with control allografts; however, it was unclear if the two groups of allografts contained the same amount of total Mn SOD (67). In addition to increased nitration, the chronically rejected allografts showed decreased Mn SOD activity, which suggests that nitration inhibits Mn SOD activity (67). Consistent with this hypothesis, Mn SOD is inactivated by peroxynitrite treatment in vitro. Furthermore, $ONOO^-$ specifically nitrates only one tyrosine residue, Tyr34, located near the bound manganese (81). Nitration of Mn SOD, responsible for scavenging $O2^{\bullet -}$ in the mitochondria, may cause mitochondrial dysfunction under inflammatory conditions such as those associated with chronic organ rejection.

Another protein known to be nitrated in vivo is the low-molecular-weight neurofilament subunit protein (82). This protein isolated from the cervical spinal cords of ALS patients was found to be nitrated. Nitration was associated with neurofilament-assembly derangement in ALS patients investigated by Chou et al (83). However, Strong et al found no differences in the quantity or quality of neurofilament nitration in familial ALS patients compared with control patients (82). Thus, the functional consequence of neurofilament nitration remains unclear.

As mentioned above, the SERCA2a isoform of the skeletal muscle sarcoplasmic reticulum ATPase, found predominantly in slow-twitch muscle, is nitrated in vivo (84). SERCA2a, which has a relatively long protein half-life, has been shown to accumulate nitrotyrosine with biological aging in a rat model (84). This accumulation of nitrotyrosine is associated with decreased Ca-ATPase activity. In vitro treatment of SERCA2a with peroxynitrite results in both increased tyrosine nitration and decreased Ca-ATPase activity. Both the in vitro and in vivo data suggest that nitration of SERCA2a inhibits Ca-ATPase activity. Tyrosine nitration is localized to Tyr 294 and Tyr 295 by tryptic digestion and V8 protease treatment of the protein followed by analysis for nitrotyrosine content. In contrast to the SERCA2a isoform of the SR CaATPase, the SERCA1 isoform, found predominantly in fast-twitch muscle, is not nitrated in vivo. The preferential nitration of the SERCA2a isoform over SERCA1a suggests that tyrosine nitration is at least somewhat selective (50).

Exposure of pulmonary surfactant protein A (SP-A) to nitrating agents such as peroxynitrite or tetranitromethane causes tyrosine nitration of this protein (85). Nitration of SP-A results in decreased mannose-binding ability and decreased ability to aggregate lipids (86). Sequencing of nitrated SP-A tryptic peptides showed that

the protein is specifically nitrated on two tyrosine residues, Tyr 164 and Tyr 166, in the carbohydrate recognition domain (87). Although to our knowledge specific in vivo nitration of SP-A has not been shown, increased iNOS activity and increased tyrosine nitration in the airway epithelium of asthmatic patients and patients with adult respiratory distress syndrome have been demonstrated histologically (85, 88).

In addition to modification of the proteins mentioned above, tyrosine nitration may also function in cellular signaling. We and others have hypothesized that nitration of tyrosine residues in tyrosine kinase substrates may prevent phosphorylation and therefore inhibit tyrosine kinase signaling (52, 89). Using a synthetic peptide, Gow and his colleagues showed that peroxynitrite-mediated nitration of the tyrosine residue in this peptide resulted in about 50% inhibition of tyrosine phosphorylation by the tyrosine kinase, c-src (52). In addition, Kong and his coworkers showed that peroxynitrite treatment of the pentadecameric peptide cdc(6-20)NH2, which corresponds to the tyrosine phosphorylation site of p34cdc2 kinase, increased nitration and inhibited phosphorylation of the peptide compared with untreated peptide (89). Crosstalk between NO and other signaling cascades has been the subject of numerous publications in recent years. The inhibition of tyrosine kinase signaling by tyrosine nitration represents a novel mechanism of NO interaction with tyrosine kinase signaling. However, to our knowledge, no tyrosine kinase substrate has been shown to be nitrated in vivo. Furthermore, in some systems, treatment with peroxynitrite has been shown to increase tyrosine phosphorylation (90, 91). Thus, the inhibition of tyrosine phosphorylation by tyrosine nitration remains highly speculative.

INTERACTION OF NO WITH TRANSITION METALS

The physico-chemical properties of NO govern its interaction with transition metals. NO can form a σ-bond through its nitrogen pair of electrons and a π-bond through the antibonding $2p\pi^*$ unpaired electron with the d-electrons of transition metals (92), acting as a three electron donor.

Effect of NO on Heme Proteins

Iron is by far the most abundant transition metal in biological systems. Not surprisingly, investigators have given much attention to the interaction of NO with iron and to the biological function of this interaction. The geometry of heme determines the character of the NO-heme interaction. As a rule, NO does not interact efficiently with six-coordinated heme (93) and has a limited effect on the function of these proteins. The character and the outcome of the NO interaction with iron depends on various factors, including the oxidation state of the iron (ferrous or ferric), the microenvironment in the heme-binding pocket (residues surrounding the prosthetic group), and the availability of oxygen or other radical species.

Soluble guanylyl cyclase (sGC) is a heterodimeric hemoprotein that converts guanosine 5′-triphosphate (GTP) into cGMP and pyrophosphate. The enzyme has

some residual basal activity in the resting state but is activated up to 500-fold upon interaction of the ferrous iron of the enzyme's heme moiety with NO (94, 95). The activation of sGC has diverse physiological effects in cardiovasculature, platelet function, neurotransmission, and other cellular aspects (18). sGC is activated by NO at a fairly low concentration (10–100 nM). Many researchers regard the effective NO concentration as crucial in determining whether NO effects are physiological or pathophysiological. However, the effective concentrations of NO in vitro or the concentrations of exogenously added NO donors in tissue cultures do not always reflect the local effective concentration of NO. In addition, the effective concentration of NO can be both positively and negatively influenced by the presence of other radicals or reactive oxygen species. The low effective concentration of NO, in the case of sGC, reflects the high affinity of NO for the sGC heme moiety. The on-rate of NO-heme binding for sGC is rather fast, in the order of 10^7–10^8 $M^{-1}\,s^{-1}$ (96, 97), which is in the same range as many ferrous-heme proteins (98). The geometry of NO binding to ferrous heme is similar to oxygen binding. Both NO and O_2 bind in a 130–150° angle towards the ligand-iron axis. NO binding to sGC disrupts the bond between the iron and coordinating His105 residue of sGC (94, 99), which may be involved in the catalytic process. The dissociation rate of NO from sGC depends on the state in which the enzyme is present. Early measurements indicated that the iron-heme complex is rather stable (93), in contrast with the biological function of sGC as a fast regulator (93). However, in the presence of GTP and Mg^{2+} the dissociation rate is increased at least 100-fold (96, 97), which indicates that the heme microenvironment changed. This is corroborated by the disturbance in the Raman spectrum of the NO-iron bond (100) upon treatment with GTP. Thus, the activation and deactivation of sGC are both likely to be very fast processes, which is in agreement with the rapid increases and decreases in cGMP levels after hormonal activation of NO/sGC (101).

Another well-documented effect of NO at low concentrations is the inhibition of the terminal complex IV (cytochrome oxidase) from the mitochondrial respiratory chain. NO-dependent inhibition of the respiratory chain is regarded as one mechanism of macrophage-derived cytotoxicity. Cytochrome c oxidase (CcO) is a complex of 13 subunits, containing 2 hemes (cyt *a* and cyt a_3) and 2 copper centers (CuA and CuB). All four redox-active metal centers have different functions in CcO catalytic activity. Low concentrations of NO cause immediate inhibition of oxygen consumption (102–104). NO acts as a potent, rapid, and reversible inhibitor with a half-inhibitory concentration in the range of 60–270 nM (104), depending on the oxygen concentration. The binding of NO to the reduced cyt a^3 is very fast and comparable to oxygen binding, with a rate of $0.4–1.0 \times 10^8$ $M^{-1}\,s^{-1}$ (105). The dissociation of NO is also rapid ($0.13\,s^{-1}$) (106). The exact mechanism of CcO inhibition by NO is not completely defined. Some authors have argued that the rapid inhibition could be explained entirely by NO binding to the reduced cyt a_3 site (106), which is also the site of O_2 binding. Others have suggested that NO binds to the CuB site, which gives NO advantage over oxygen in binding to cyt a_3 (107). The formation of a stable Cu^{2+}-NO complex as a mechanism of CcO inhibition has also been

proposed (108). NO also reacts with the oxidized CcO; however, it has been suggested that this interaction forms an irreversible bridged complex, a_3^{2+}-NO-Cu_B^{2+}, rather than a reversible complex, a_3^{3+}-Cu_B^{2+}-NO, after NO binding to reduced CcO (109). Although inhibition of CcO with NO occurs in the physiological range of NO concentration, the biological significance of such inhibition has not been clarified. Some researchers hypothesize that the balance between intracellular levels of O_2 and NO dictate the ultimate respiratory rate of the mitochondria (110).

Catalase is a ferric heme protein that is critical for intracellular degradation of hydrogen peroxide. As a rule, NO binds less tightly to ferric iron (98); however, when the iron coordination with the distal ligand is weak or absent, as in the case of catalase, the on-rate for NO can be reasonably fast:$3 \times 10^7\ M^{-1}\,s^{-1}$ (111). NO binding to ferric heme is less reversible, which in the case of catalase results in the NO-dependent inhibition of the enzyme with a K_i of 0.18 uM (112). An increase in NO production or addition of NO-donors results in a decrease of cellular hydrogen peroxide consumption (113, 114). Although the role of catalase inhibition is unclear, NO-dependent inhibition of catalase resulting in increased hydrogen peroxide concentrations may enhance the cytotoxic effect of NO.

Nonheme Iron Proteins

NO can also react with iron in FeS clusters. The early studies of macrophage cytotoxicity demonstrated the inhibition of mitochondrial respiration (115) in the complex I and II (116) in tumor cells cocultured with activated macrophages. Changes in activities indicated that aconitases were most sensitive and were inhibited first, followed by complex I and complex II (117, 118). The richness of these complexes in [4Fe-4S] clusters designated them as primary targets of what was later shown to be a nitric oxide–mediated effect. Aconitases are a family of dehydratases that catalyze the reversible isomerization of citrate and isocitrate via cis-aconitate (119). These enzymes contain unique [4Fe-4S] clusters in which one of the irons, Fe_a, is not ligated to a protein residue but rather to a hydroxide from solvent. The substrate reacts with this apical iron. A direct NO interaction with the mitochondrial FeS clusters has been proposed, based on the detection of a paramagnetic $g = 2.03$ electron paramagnetic resonance (EPR) signal interpreted as the formation of nitrosyl-iron-sulfur complexes (120). However, the question of whether mitochondrial FeS clusters participate in the formation of a $g = 2.03$ complex [also referred to as dinitrosyl-iron-dithiol (DNIC)] remains unsettled.

The cytosolic form of aconitase is a bifunctional protein, carrying a [4Fe-4S] cluster that acts as a regulator of enzyme function (121). As a holoenzyme form, the enzyme has aconitase activity, which can be inhibited by NO-treatment and protected by the presence of the enzyme's substrate, citrate. This suggests a direct interaction of NO with the apical iron, Fe_a. In an apoenzyme form, lacking the [4Fe-4S], the enzyme acts as a posttranscriptional regulator, known as iron regulatory protein (IRP) (122). IRP acts as a translational regulator of the intracellular iron pool. IRP binds to the iron response element (IRE) of the 5′ untranslated

regions of ferritin and erythroid δ-aminolevulinilate synthase to attenuate their translation (123) or to the 3′ region of the transferrin receptor to stabilize its mRNA (124). In the iron-depleted cells, the IRP is in the opened apo-conformation and binds to RNA, whereas in the iron-supplied cells the IRP retains its [4Fe-4S] cluster and acts as an aconitase (125). The activated macrophages produce NO, exhibit the $g = 2.03$ EPR signal, and display a decrease in aconitase activity and an increase in IRE-binding activity of IRP (126, 127). Thus, it is compelling to assume that NO has a direct effect on the conversion of [4Fe-4S]-containing cytosolic aconitase into a [4Fe-4S]-deficient IRP as a result of nitrosylation of the apical Fe_a. However, there is little evidence indicating that the direct NO binding to aconitase strips it of its [4Fe-4S]-cluster and converts it directly to IRP. Although the direct binding of NO to the apical Fe_a converts aconitase into inactive [3Fe-4S]-enzyme, these forms of aconitase do not bind IRE (125), which suggests that NO-dependent modulation of aconitase-IRP conversion can either be nondirect or involve more potent reactive nitrogen species. It is also possible that NO prevents the assembly of a [4Fe-4S]-cluster rather than dismantling of the aconitase into the iron-free enzyme. Some of the possible mechanisms are discussed in more detail in recent reviews (122, 128).

An interesting example of direct interaction between NO and FeS clusters is presented by the *Escherchia coli* SoxR activator protein. SoxR is a 17-kDa DNA-binding factor regulating the expression of the SoxS protein, which activates the transcription of all protein members of the oxidative stress *sox*RS regulon. The upregulation of proteins by the SoxRS regulon results in increased resistance to oxidative stress. SoxR protein forms a homodimer containing a [2Fe-2S] cluster. This cluster exists in the reduced state under normal aerobic growth, but can be oxidized upon cell exposure to a number of agents, including superoxide or NO (129, 130). Oxidation by NO or superoxide switch the protein into an activation mode, which leads to a 100-fold stimulation of soxS expression (131, 132). SoxR protein treated with NO displays a $g = 2.03$ EPR spectrum (130, 133) typical for dinitrosyl-iron-dithiol complexes (120). NO activation of the *soxRS* regulon results in an increased bacterial resistance to activated macrophages (133) and represents one of the potential defensive mechanisms of bacterial cells against oxidative stress in general and NO-mediated toxicity in particular.

Ferritin, a protein crucial in the regulation of cellular iron pool availability, displays three types of EPR signals attributed to iron-nitrosyl complexes at imidazole groups of histidine, thiol groups of cysteine, and carboxylate groups of aspartate and glutamate (134). When the reaction between NO, apo-metallothionein (or Zn-metallothionein), and iron was examined by electron spin resonance spectroscopy, paramagnetic products with g values of 2.013 and 2.039 were detected. These EPR spectra are similar to dinitrosyl-iron-dithiol complexes (135). However, it is still not clear whether this in vitro treatment with NO reflects the physiological or pathophysiological events taking place under NO stress. The modification of ferritin by NO may be an important step in the NO-dependent regulation of the intracellular iron pool.

Interaction of NO with Other Transition Metals

The role of biological Zn is best characterized for the Zn-finger proteins. Zn is coordinated mainly by conserved cysteine and histidine residues. Nearly 1% of human genes may encode Zn-finger proteins (136). Other Zn-chelating structures have also been described (for review see e.g. 137). The effect of NO on the function of Zn-finger-containing nuclear receptors was recently reported (138). The specific interaction of the heterodimeric complex of two Zn-finger transcription factors, 1,25-dihydroxyvitamin D3 [1,25(OH)2D3] receptor (VDR) and retinoid X receptor (RXR) with 1,25(OH)2D3 response elements (VDREs), was used as a model system. NO caused a dose-dependent inhibition of VDR-RXR-VDRE complex formation (138). It should be noted, however, that these in vitro effects were observed at a rather high concentration of NO (IC50 values 0.5–0.8 mM). In vitro treatments of the Zn-finger containing DNA repair enzyme formamidopyridine-DNA glycolyase (Fpg) resulted in inactivation of Fpg (135a). The enzyme was protected, if free cysteine was present during incubations, suggesting that the cysteine-residues coordinating Zn may be the primary targets of NO and RNS rather the Zn-ion itself. Considering the staggering amount of proteins potentially containing coordinated Zn (136) and their function as regulators of expression, such NO-dependent changes of their properties could have an important role in the regulation of transcription under NO stress.

In recent years, interesting data have emerged that demonstrate a rather complicated crosstalk between nitric oxide/RNS and the tumor suppressor p53. p53 is a transcriptional regulator that has a unique DNA-binding domain structure. The DNA-binding domain is made up of an array of two-beta sheets supporting large loop-helix structures directly involved in contacting DNA (139). These loops are bridged together by the coordination of the divalent Zn atom with three cysteines and one histidine. Expression of p53 in a variety of human cell lines and in murine fibroblasts downregulates the transcription from the NOS2 promoter, which suggests a negative feedback loop that protects cells from NO-induced damage (140, 141). However, NO donors have also been shown to induce conformational changes in p53, resulting in the inactivation of the DNA-binding properties of the protein (142). Although the exact nature of these NO-dependent changes has not been elucidated, nitrosylation of the Zn ion is one possible mechanism. Such disruption of p53 function by NO may represent a mechanism for its inactivation in some cancer or precancer conditions.

INTERACTION OF NO WITH RADICAL RESIDUES

Reaction with Protein Radicals

NO is a paramagnetic molecule that is capable of reactions with other radicals. The interaction of NO with $O_2^{\bullet -}$ and other free radical molecules has a significant biological importance, as discussed elsewhere in this review. A vast number of

biochemical reactions proceed via radical intermediates. Some of these reactions utilize tightly controlled protein-bound amino acid radical residues as cofactors. The amino acid involved can be a tyrosine (ribonucleotide reductase class I, photosystem II, prostaglandin H synthase), a modified tyrosine (amine oxidase, galactose oxidase), a tryptophan (cytochrome c peroxidase), a modified tryptophan (methylamine dehydrogenase) or a glycine (ribonucleotide reductase class III, pyruvate formate lyase) (for review see 143). Such protein radicals are likely targets for NO and RNS.

Ribonucleotide Reductases

Ribonucleotide reductases (RNRs) catalyze the reduction of ribonucleotides to deoxyribonucleotides crucial for the synthesis of DNA. The class I RNR requires a tyrosyl radical for its enzymatic activity. The tyrosyl radical of RNR is stabilized owing to its delocalization over the aromatic ring and its position in the protein core with the lack of oxidizable amino acid side chains in close proximity (144, 145). The tyrosyl radical is magnetically coupled with a pair of nonheme iron atoms involved in the production and stabilization of the radical (146). Although the diferric center of RNR is not very sensitive to NO (147), the enzyme is rather susceptible to NO (148, 149), presumably owing to NO scavenging of the tyrosyl radical (150). It was estimated that 0.5–3.0 uM NO scavenge 100% of the RNR tyrosyl radicals available in mammalian cells (151). This amount of NO can be attained through activation of iNOS. The inhibition of RNR by NO results in inhibition of DNA synthesis and is widely regarded as one of the important mechanisms of macrophage-dependent cytotoxicity.

Prostaglandin H Synthase

Prostaglandin H synthases (PGHS) (cyclooxygenases) catalyze the first two steps in the biosynthesis of prostanoids. The involvement of the tyrosyl radical of PGHS in catalysis was demonstrated by EPR studies (152) and later confirmed by other methods (153, 154). The PGHS tyrosyl radical is considerably less stable [half-life $=$ 20 s at -12°C (152)] compared with the RNR tyrosyl radical. The interaction between NO and PGHS tyrosyl radical was documented only recently by low-temperature EPR spectroscopy and by the presence of nitrotyrosine modification of the catalytically active tyrosine residue (62, 155). In contrast to ribonucleotide reductase, RNS may activate PGHS (156). Some investigators showed that PGHS-1 is inhibited by NO donors but stimulated by compounds capable of generating peroxynitrite (157).

NO MODIFICATIONS OF DNA

NO is probably insufficiently reactive to attack DNA directly, but numerous RNS and their CO_2^- or Cl^- adducts can oxidize, nitrate, or deaminate genomic DNA, resulting in strand breaking and mutations (158–160). It is unlikely that O_2, $O_2^{\bullet -}$,

and H_2O_2 are capable of reacting directly with DNA, at least at their physiological levels, but the hydroxyl radical ($HO^{\bullet}$) derived from H_2O_2 generates a multiplicity of products from all four DNA bases (158–160). However, the physiological role of $HO^{\bullet}$ is uncertain because of its extremely high reactivity. Because $HO^{\bullet}$ production in living cells requires the presence of a transition metal (such as Fe^{2+}), it could damage DNA only when the transition metal is immediately adjacent to the DNA. Because metal ions appear to be mainly in a form that is unable to catalyze free radical reactions, their availability for reaction with $HO^{\bullet}$ is restricted in vivo. In contrast, peroxynitrite can travel up to 9 μm (161) and easily pass through biological membranes (162). Thus, peroxynitrite is more likely to modify DNA than $OH^{\bullet}$.

Studies have shown that peroxynitrite preferentially reacts with guanine, but because of the various reactions that peroxynitrite can undergo, there are also a variety of products observed. The reaction of peroxynitrite with guanine yields 8-oxo-deoxyguanosine (8-oxo-dG), one of the most abundant products of DNA oxidation by peroxynitrite (163). 8-oxo-dG is known to cause GC $\rightarrow$ TA transitions, mispairing, and ultimately, mutations. Once 8-oxo-dG is formed, it becomes approximately 1000-fold more reactive with peroxynitrite than its precursor, dG (164). Depending on the ratio of peroxynitrite over 8-oxo-dG, the oxidation reaction may continue until the oxidized product, oxaluric acid, is formed (165). Cyanuric acid, oxazolone, and 4-hydroxy-8-oxo-4,8-dihydro-2′-deoxyguanosine may also be generated as a result of secondary oxidation of 8-oxo-dG (158). 8-oxo-dG is formed by many DNA-damaging agents in vivo, as well as by $ONOO^-$. In contrast, 8-nitro-dG formed by the reaction of peroxynitrite with DNA is more specific for peroxynitrite-induced damage to DNA. Recently, another protein-nitrating agent, nitryl chloride, has been reported (166). However, it is unclear whether nitryl chloride can nitrate guanine in the same manner as peroxynitrite.

Peroxynitrite treatment also causes DNA strand breaks. Generally, peroxynitrite can abstract hydrogen from sugar moieties and form sugar radicals leading to sugar fragmentation and DNA strand breaks (167). The formation of 8-nitro-dG by reaction with peroxynitrite may also favor the creation of basic sites favorable for cleavage by endonucleases

Another powerful reactive nitrogen species, N_2O_3, acts as a nitrosating agent. N_2O_3 may damage DNA directly through the nitrosation of primary amines on DNA bases or indirectly through reactions with primary and secondary amines, ultimately leading to DNA deamination. This chemistry was recently reviewed in detail (158). Deamination is the replacement of an exocyclic amino group by a hydroxyl group. Therefore, any DNA base containing such an amino group can be deaminated in the reaction with N_2O_3. The major consequences of this deamination reaction are GC $\rightarrow$ AT, GC $\rightarrow$ TA, and AT $\rightarrow$ GC transitions and single-strand breaks (168, 169). N_2O_3 may also cause intra- and interstrand cross-links that, even formed in very small amounts, disrupt gene expression.

Direct chemical modification of DNA by reactive nitrogen species derived from NO may be an important contributor to the age- and inflammation-related

development of cancer or other diseases. There are three major techniques used to identify and quantify oxidized DNA products: gas chromatography/mass spectroscopy with selected ion monitoring (170), reverse phase HPLC with electrochemical detection (171), and liquid chromatography/electrospray ionization mass spectrometry (165). Each of these techniques may produce artifacts that have created some discrepancy in the field, especially in terms of quantities of oxidized DNA products. These discrepancies have also raised the question—how high is the steady-state level of DNA damage in normal and pathological conditions? Keeping in mind the existence of nonexpressed DNA and well-balanced DNA repair systems, the contribution of DNA oxidation to age-related development of cancer is unclear. Despite debatable quantitative issues, 8-oxo-dG generation was used for years to monitor DNA damage resulting from treatment with various chemical and physical agents. Although there is no direct evidence that 8-oxo-dG causes cancer, several studies have shown a correlation between elevated levels of 8-oxo-dG and carcinogenesis (172–174). It is generally accepted that the high frequency of point mutations in certain common human tumors can be induced by exposure to reactive nitrogen species. It was shown that excess production of NO in chronic inflammation causes DNA damage, inhibits DNA repair, and may link inflammation and cholangiocarcinoma (175). RNS production has been linked to human colon adenomas and carcinomas (176) and to breast (177) and gastric (178) cancer. Reactive nitrogen species can also cause mutations in cancer-related genes, such as tumor suppressor gene p53 (179).

In addition to nuclear DNA, there is also mitochondrial DNA (mtDNA). Mammalian mtDNA codes 13 subunits of respiratory chain complexes and its own structural rRNAs and tRNAs. MtDNA is a 16,569-bp double-stranded circular DNA that is mutated much faster than nuclear DNA, presumably because mtDNA is not protected by organization along histones. It has no introns, and a random hit will inevitably cause damage or mutation with serious consequences. It is important to note that mtDNA contains unusually high amounts of direct repeats that may give rise to large-scale deletions by mispairing during oxidized DNA replication. So far, more than 50 pathogenic mtDNA mutations have been found that are associated with or responsible for specific human diseases (159, 180, 181). Five different types of mtDNA mutations were found: deletions, point mutations, insertion, tandem duplications, and DNA rearrangements. This broad spectrum of mutations of mtDNA accumulates in various human tissues and accompanies aging. Because mtDNA is located in the vicinity of the reactive oxygen species generation site in the mitochondrial inner membrane, investigators hypothesized that reactive oxygen species–associated oxidation causes damage to this genome (180). However, recent evidence that mitochondria may be a significant intracellular source of peroxynitrite may to a large degree refocus research. The existence of a mitochondrial NOS has been reported for several rat tissues (182, 183). The actual production of NO in mitochondria has also been demonstrated (184, 185). Furthermore, NO can diffuse freely and is more soluble in organic phases than in water. Mitochondria are also the major source of $O_2^{\bullet -}$ production in cells. Any

perturbation capable of decreasing the coupling efficiency of the mitochondrial respiratory chain generates $O_2^{\bullet-}$. The well-known reaction between NO and $O_2^{\bullet-}$ generates $ONOO^-$; however, $ONOO^-$ may also be formed by the reaction of nitroxyl anion (NO^-) with $O_2^{\bullet-}$ (186). Recently, it was reported that cytochrome c can catalyze the reduction of NO to NO^- (187), which raises the possibility that the formation of NO^- may be an important source for the production of $ONOO^-$ in mitochondria.

Recent studies indicate that oxidative damage of mtDNA results in fragmentation (188). However, the level of the oxidized bases in full-size mtDNA, the only template for replication, is rather low. This finding indicates that, despite the extensive mtDNA oxidation, efficient mtDNA repair and degrading systems decrease accumulation of negative changes. Although the general consensus is that mtDNA is subject to severe oxidative damage, the critical questions of what type of reactive species participate in this damage and what kind of contribution oxidative damage makes to human diseases and aging remain to be addressed.

NO MODIFICATIONS OF LIPIDS

Numerous mechanisms of lipid oxidation, both enzymatic and nonenzymatic, have been thoroughly studied in vitro. It is also well established that lipid oxidation is a typical feature of inflammatory diseases. However, there is a very little understanding of the oxidative mechanisms in vivo and how they impinge upon lipid-mediated signal transduction, integrity, and fluidity of biological membranes, and enzymatic properties. Identification of specific oxidants that are responsible for lipid oxidation in inflammatory conditions remains a major priority.

Reactive oxygen species derived from NO may interact with unsaturated lipids (189). Chemical mechanisms for these reactions generally fall into two categories: oxidation and nitration. A complicated and incompletely understood set of factors determines which lipid oxidation products are generated in vivo in each particular case. However, it is clear that the role of NO is different in the presence of $O_2^{\bullet-}$ compared with the absence of $O_2^{\bullet-}$ (22).

In the absence of $O_2^{\bullet-}$, NO may terminate lipid oxidation (190). Several mechanisms for this termination have been described, namely (*a*) NO trapping of alkyl, aloxyl, and peroxyl lipid–derived radicals; (*b*) NO regulation of the activity of enzymes such as cyclooxygenase, lipoxygenase, and cytochrome P-450; (*c*) NO regulation of cell signaling not directly associated with lipid oxidation; and (*d*) NO binding to redox-active metal centers, which inhibits metal-catalyzed $HO^{\bullet}$ generation and lipid peroxidation.

In the presence of $O_2^{\bullet-}$, the NO concentration is decreased and a variety of oxidant and nitrating agents are formed. These nitrating and oxidizing agents react with lipids to form several oxidation products, which may be subsequently nitrated to form different products. Because O_2 may also react with lipid oxidation products, the concentration of O_2 may determine whether nitration occurs (191).

In vivo the high concentration of thiols may successfully compete with lipids for reactive nitrogen species.

$ONOO^-$ is one of the major nitrating species. $ONOO^-$ may be formed in either the aqueous or the hydrophobic phase of the cell. Presumably, peroxynitrite undergoes different chemical reactions that contribute differently to lipid oxidation in these two compartments. It is well established that $ONOO^-$ rapidly permeates model phospholipid membranes in vitro (162) and moves through intact erythrocyte or mitochondrial membranes (192). Because $ONOO^-$ reacts rapidly with many substances, it is not clear how much peroxynitrite, formed in the aqueous phase, contributes to lipid oxidation.

Regardless of the source of reactive nitrogen species, the hydrophobic phase has been shown to be more favorable for their reactions than the aqueous phase (54, 162, 193). It was shown that NO is more soluble in the hydrophobic phase (194), and the reaction of NO with O_2 within membranes is approximately 300 times more rapid than in the surrounding aqueous medium (193). The membrane permeability coefficient for peroxynitrite is close to that reported for water (195). Therefore, peroxynitrite can be expected to have free access to hydrophobic compartments in cells and to hydrophobic structures like atherosclerotic plagues, myelin sheaths, or the lining of the lung. The life span for peroxynitrite is expected to be longer in the hydrophobic phase than the aqueous phase. Thus, its capacity to accumulate in the hydrophobic phase can play a critical role in regulating membrane and lipoprotein lipid oxidation reactions.

Besides oxidation and nitration, other reactions of reactive nitrogen species with lipids that may occur in vitro include decarboxylation of free fatty acids (196). Reactions with aliphatic or aromatic alcohol groups, resulting in alkyl or aryl nitrites, are also possible. Because these reactions are rather slow, their physiological relevance is unclear.

Oxidation and nitration convert low density lipoproteins into an atherogenic high-uptake form (197). It has been shown (198, 199) that a variety of halogenating and nitrating intermediates may be generated by the myeloperoxidase-H_2O_2-Cl^- system in the presence of nitrite (NO_2^-), the autoxidation product of NO. Their potential roles are still obscure. The latest statement is that nitrating intermediates are more effective than chlorinating intermediates in promoting oxidative conversion of LDL into a stable high-uptake form (197). However, the biological consequences of oxidative modification of LDL, as well as the precise oxidative and nitrating intermediates, remain to be established.

MISCELLANEOUS

Various antioxidants may react with peroxynitrite and inhibit peroxynitrite-mediated oxidation and nitration reactions (200). The reported list includes ascorbate (201, 202), uric acid (203), bilirubin (204), vitamin E (205), catecholamines (206), flavonoids (207), melatonin (208), glutathione (209), and β-carotene (210).

Although the apparent rates of these reactions are not very fast, they may occur in vivo and accelerate $ONOO^-$ decay. For example, ONOO-mediated hemolysis is effectively inhibited by glutathione (211).

Another point of interest is the generation of stable NO donors in peroxynitrite-dependent reactions. For example, peroxynitrite can react with compounds containing an alcohol functional group, e.g. D-glucose (212). This could be an additional pathway for peroxynitrite decay. However, this also could represent an additional way to recycle reactive nitrogen species and generate compounds with cytoprotective properties, namely longer-lived NO donors. Formation of low–molecular weight nitrosothiols probably falls in this category.

Summary

The chemistry of NO, with its diverse array of modifications and products, is obviously complex. This unique free-radical gas can participate in cellular signaling and regulation in a variety of ways, which we have attempted to summarize in this review. The reactions of NO, and consequently the effects elicited by NO, are a function not only of the concentration and location of NO but also the surrounding milieu in which NO is produced. NO or the products of the reaction of NO with O_2 and $O_2^{\bullet -}$ can modify many different macromolecules, including proteins, lipids, and nucleic acids, to produce both physiological and pathophysiological effects. Although there are more than 30,000 publications on NO, numerous questions remain unanswered.

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