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Review Article

The Activation of Metabolites of Nitric Oxide Synthase by Metals Is Both Redox and Oxygen Dependent: A New Feature of Nitrogen Oxide Signaling

SONIA DONZELLI,[1] CHRISTOPHER H. SWITZER,[1] DOUGLAS D. THOMAS,[1] LISA A. RIDNOUR,[1] MICHAEL GRAHAM ESPEY,[1] JEFFREY S. ISENBERG,[2] CARLO G. TOCCHETTI,[3] S. BRUCE KING,[4] GIUSEPPE LAZZARINO,[5] KATRINA M. MIRANDA,[6] DAVID D. ROBERTS,[2] MARTIN FEELISCH,[7] and DAVID A. WINK[1]

ABSTRACT

Nitrite (NO_2^-), N^G-hydroxy-L-arginine (NOHA), and hydroxylamine (NH_2OH) are products of nitric oxide synthase (NOS) activity and can also be formed by secondary reactions of nitric oxide (NO). These compounds are commonly considered to be rather stable and as such to be dosimeters of NO biosynthesis. However, each can be converted *via* metal-catalyzed reactions into either NO or other reactive nitrogen oxide species (RNOS), such as nitrogen dioxide (NO_2) and nitroxyl (HNO), which have biologic activities distinct from those of the parent molecules. Consequently, certain aspects of tissue regulation controlled by RNOS may be dictated to a significant extent by metal-dependent reactions, thereby offering unique advantages for cellular and tissue regulation. For instance, because many metal-catalyzed reactions depend on the redox and oxygen status of the cellular environment, such reactions could serve as redox indicators. Formation of RNOS by metal-mediated pathways would confine the chemistry of these species to specific cellular sites. Additionally, such mechanisms would be independent both of NO and NOS, thus increasing the lifetime of RNOS that react with NO. Thus metal-mediated conversion of nitrite, NOHA, and NH_2OH into biologically active agents may provide a unique signaling mechanism. In this review, we discuss the biochemistry of such reactions in the context of their pharmacologic and biologic implications. *Antioxid. Redox Signal.* 8, 1363–1371.

INTRODUCTION

Since he discover in the 1980s that nitric oxide (NO) regulates vascular tone by activating the heme protein soluble guanylyl cyclase (10, 14, 15, 46, 52, 63, 77, 78, 90), NO has been implicated as a mediator of nearly every major mammalian physiological system (81) as well as a participant in a variety of pathophysiologic conditions (13, 16, 23, 87). Accordingly, clarification of the biologic functions of nitrogen oxides has been the subject of a significant fraction of biomedical research over the past two decades. Fundamental to this process is the characterization of the chemical biology

[1]Tumor Biology Section, Radiation Biology Branch, National Cancer Institute, NIH, Bethesda, Maryland.
[2]Biochemical Pathology Section, Laboratory of Pathology National Cancer Institute, NIH, Bethesda, Maryland.
[3]Division of Cardiology, Department of Medicine, Johns Hopkins Medical Institutions, Baltimore, Maryland.
[4]Department of Chemistry, Wake Forest University, Winston-Salem, North Carolina.
[5]Department of Chemical Sciences, Laboratory of Biochemistry, University of Catania, Catania, Italy.
[6]Department of Chemistry, University of Arizona, Tucson, Arizona.
[7]Whitaker Cardiovascular Institute, Boston University School of Medicine, 650 Albany Street, Boston, Massachusetts.

of NO, which begins with enzymatic formation of NO by NO synthase (NOS), progresses to the reactivity of NO, continues with the chemical modifications of reactive nitrogen oxide progeny, and concludes with the effects of nitrogen oxide–induced alterations of biomolecules on physiology and pathophysiology. This description emphasizes the tenet that in mammalian systems, NO is the principal species from which all other endogenous nitrogen oxides arise. However, the importance of nitrogen to biology has long been understood to originate from its capacity to exist in all oxidation states from +5 (NO_3^-) to –3 (NH_3). This review is concerned with nitrogen oxides other than NO that are produced as a result of oxidation of L-arginine by NOS.

Under normal physiologic conditions, the constitutive NOS isoforms [eNOS or NOS-3 and nNOS or NOS-1 (53,94)] produce relatively low concentrations of NO (<1 μ*M*). Upregulation of inducible NOS (iNOS or NOS-2) expression on activation of the immune system or under disease states results in considerably higher levels of NO. Because the concentrations of nitrite and nitrate produced on iNOS induction exceed basal levels, which are relatively constant (62), nitrite and nitrate have been assumed to be stable metabolites of NO and thus to be reliable indicators of NO biosynthesis (40).

Formation of NO by NOS involves a two-step oxidation of L-arginine to L-citrulline through the intermediate *N*-hydroxy-L-arginine (NOHA) (20, 35, 60, 64, 76, 99). Rather than catalyzing the complete oxidation of L-arginine, iNOS releases a considerable fraction of NOHA, such that its steady-state concentration can approach 30% of all NOS products (14, 46, 94). Examination of the end products of NOS has also revealed the generation of substoichiometric amounts of NH_2OH (90).

Although nitrite, NOHA, and NH_2OH are generally thought to be relatively stable and consequently to function primarily as dosimeters of NOS activity, each can be activated to form several different reactive nitrogen oxide species (RNOS) in a manner independent of either NOS and NO. Additionally, recent analyses (12) suggest that nitrite itself can function as a signaling molecule and regulator of gene expression in its own right. Such processes may provide important alternative mechanisms through which redox signaling is controlled in cells and tissues.

Nitrite

Nitrite can be either ingested (72) or derived from autoxidation of NO (50) or from reduction of dietary nitrate by salivary bacteria (68). Nitrite is stored in tissue and is found in various fluids including blood, urine, saliva, and cerebral spinal fluid (CSF). Studies suggest that cells such as red blood cells take up nitrite and thus facilitate nitrite transport (24). Because nitrite is converted to nitrate by oxyhemoglobin (25, 41), plasma levels of nitrite [0.2–2 μ*M* (12)] are considerably lower than those in nonheme containing fluids [*e.g.*, 5–10 μ*M* in CSF (13, 17, 42, 87, 88)].

Nitrite administered by intraperitoneal injection is rapidly absorbed and distributed systemically (12). The increase in nitrite levels in tissue is accompanied by a slow conversion of nitrite to nitrate and increased levels of *S*-nitrosothiols (RSNOs) and metal nitrosyls in every tissue examined except for the aorta and brain. These results suggest that nitrite pools are tightly regulated and that such regulation has chemical consequences. Nitrite is known to be consumed by four pathways: metal-mediated oxidation, metal-mediated reduction, acidification, and direct reaction with biotarget, with the first three reactions resulting in RNOS formation.

REDOX-SENSITIVE CONVERSION OF NITRITE TO THE FREE RADICALS NO AND $NO_2^{\bullet}$

Under hypoxic conditions, tissue levels of nitrite decrease by as much as 75%, with concomitant although nonstoichiometric increases in metal nitrosyl complexes and RSNO levels (13). Such conversion indicates that nitrite may have a physiologic role under hypoxic conditions. The products suggest that nitrite serves as a secondary, or perhaps even primary, source of NO under hypoxic conditions because NOS is an O_2-dependent enzyme (38, 101). That tissue has the capacity of reducing nitrite to NO was verified in humans in a seminal study in which administration of nitrite was demonstrated to induce vasodilatation, particularly under hypoxic conditions (23). The conversion of nitrite to NO may be involved in the maintenance blood flow through tissue, even during normal muscle work. Additional studies showed that nitrite could alleviate the effects of cerebral vasospasms (85) and can attenuate ischemia–reperfusion injury in the heart and liver (29, 100). Consequently, nitrite may have pharmacologic potential for the treatment of hypoxic stress via release of the antioxidant NO and may function as an endogenous component of the stress–response mechanism.

Investigation of the effects of intraperitoneal administration of nitrite under normal conditions showed no appreciable changes in mean arterial pressure, except at the highest dose, with which a modest reduction was observed (12). However, cyclic guanosine monophosphate (cGMP) levels increased in the liver and kidney and less appreciably in the brain and aorta. Nitrite also reduces heme oxygenase levels in the aorta, inhibits cytochrome P450 activity, and elevates HSP70 activity. These results indicate that conversion of nitrite to NO may also be a critical component of signaling systems under normal physiologic conditions.

Reduction of nitrite to NO by metals is well known (31). For instance, bacterial reduction of nitrite to ammonia involves the formation of NO and less-well-defined HNO intermediates and is crucial in the global nitrogen cycle (50, 110). In the 1980s, Doyle *et al.* (26–28) demonstrated that hemoglobin can reduce nitrite to NO. The nitrite reductase functionality of hemoglobin has received current attention and has been found to be allosterically controlled. Reduction of nitrite to NO by heme deoxygenation is related to the heme redox potential, with maximal activity observed at 50% hemoglobin oxygenation (P_{50}) (23). That vasodilation via nitrite reduction is initiated at the protein P_{50} suggests that oxygen sensing by hemoglobin is mechanistically linked to nitrite reduction. Interestingly, mutation at the β93 cysteine decreased the heme-reduction potential and enhanced the reduction rate, indicating that modification of this site may influence the reduction process. Other metalloenzymes

such as xanthine oxidase may also contribute reduction of nitrite to NO (65, 100).

In addition to being reduced to NO, nitrite can be oxidized to both NO_2 and nitrate. The oxidation of nitrite into nitrate by peroxide and catalase was first reported in 1949 by Heppel and Porterfield (48). Chance (19) later reported that the rate of nitrite oxidation (2×10^3 M/s) was comparable to that of alcohols, whereas Parks *et al.* (81) demonstrated that intratracheal administration of nitrite resulted in enhanced nitrate levels as a result of oxidation by catalase. Doyle *et al.* (25) suggested that the induction of hemoglobin autoxidation by nitrite proceeds through an NO_2 intermediate. Chan *et al.* (18) indicated that NO_2 was a component of the immune system arsenal against *Mycobacterium tuberculosis* (18). As such, nitration observed under inflammatory conditions in the lung may be indicative of NO_2 formation as antipathogen response (Fig. 1).

The generation of NO_2 by the metal-mediated oxidation of nitrite may have several advantages. When NO_2 is generated during the NO/O_2^- reaction, the secondary reactions of NO_2 with O_2^- or NO results in scavenging and a reduction in oxidation (43, 95, 105). Thus, the diffusion and chemical capacity of this NO_2 is diminished when produced from NO pathways. In contrast, NO_2 produced via oxidation of nitrite by the peroxidase reaction is not inherently subject to consumption by the near diffusion-controlled secondary reactions of NO or O_2^- (32, 33, 95). This mechanism may then provide a method to maximize delivery of NO_2 to pathogens and may possibly generate NO_2 as a signaling agent (91, 92, 109).

Recent studies have shown that specific proteins are targeted for nitrotyrosine formation under inflammatory conditions (6). An intriguing study shows that tyrosine residues in mitochondrial proteins are nitrated reversibly as a function of oxygen tension (5), thus potentially providing an oxygen-sensing mechanism. Furthermore, nitrotyrosine formation parallels peroxidase expression under inflammatory conditions of macrophage activation (82, 83). This indicates that NO_2 formation in macrophages follows both the reactive oxygen species burst and the high-NO-output phases of the cytokine-activated macrophage. It is likely that high NO is attenuated by peroxidase scavenging, which should result in nitrite formation (1). When NOS is downregulated, thus reducing NO levels, nitrite can then be oxidized to NO_2 during a specific period of the inflammatory response. Drapier and co-workers (96) recently suggested that NO_2 may increase HO-1 as well as interleukin (IL)-8 levels (96), which indicates a transition from a microbial/inflammation response to a wound-healing response.

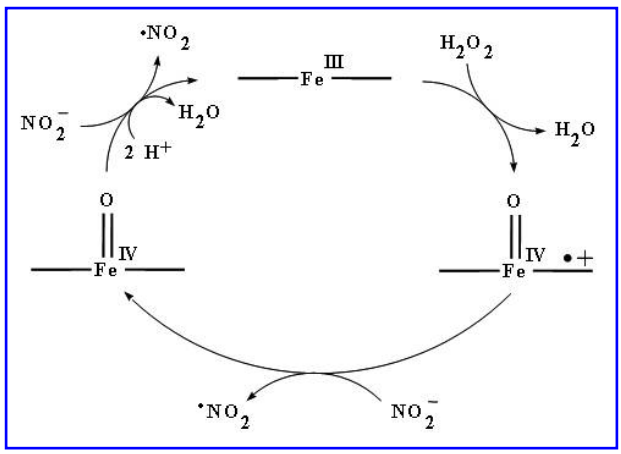

FIG. 1. Nitrite oxidation.

Under conditions of inflammation, nitrotyrosine has been used as a general indicator of RNOS, although several different mechanisms are proposed to produce this species. In 1998, nitrite was shown to generate nitrotyrosine in the presence of myeloperoxidase (MPO) (30), thereby opening the possibility that nitrite could account for the nitrotyrosine produced under inflammatory conditions and that this chemistry, as proposed by Klebanoff (61), could be part of the endogenous antimicrobial response. In support of this hypothesis, MPO knockout mice were found to have considerably lower nitrotyrosine levels than normal mice (7). Interestingly, MPO itself has been suggested to scavenge nitrotyrosine (51). That MPO knockout mice have measurable levels of nitrotyrosine (11) suggests the presence of additional mechanisms. Other heme proteins and metal complexes can oxidize nitrite to generate nitrotyrosine under oxidative-stress conditions (9, 89, 95).

Nitrotyrosine formation is often assumed to be indicative of the presence of peroxynitrite ($ONOO^-$) (98), a reactive product of the reaction of NO with O_2^- under oxidative-stress conditions. However, a high bolus dose of synthetic $ONOO^-$ can generate a yield of only ~1% nitrotyrosine (11). Nitrotyrosine formation from the NO/O_2^- reaction also is more complicated than the simple administration of synthetic $ONOO^-$ (83, 84, 95). An excess of either NO or O_2^- will react with $ONOO^-$, in effect quenching this oxidizing species and preventing nitrotyrosine formation unless the rates of NO and O_2^- production are nearly identical. Even under these conditions, the yields are quite low. Therefore it is unlikely that the NO/O_2^- reaction facilitates significant nitrotyrosine formation *in vivo* (84). Experiments using different fluxes of NO and O_2^- (generated outside or inside the cell) showed little nitrotyrosine formation inside the cell (32, 95), further supporting the generation of nitrotyrosine from NO_2, perhaps produced by metal-mediated oxidation of nitrite.

Because NO_2 also reacts rapidly with sulfhydryls, it is likely that membrane-associated cysteine-containing proteins will be molecular targets of NO_2. The oxidation of cysteine-containing peptides and proteins leads to the formation of the thiyl radical, which under aerobic conditions is oxidized to RSO_2. In the presence of thiols, such as GSH, the disulfide is the primary product, whereas in the absence of thiol, RSO_2 decomposes to sulfenic and sulfinic acid.

Metal-mediated oxidation or reduction of nitrite provides an intriguing new pathway to regulate tissue function and/or to provide a site-specific source of NO or RNOS. This chemistry is dependent on oxygen tension, with normal or oxidative-stress conditions resulting in the formation of H_2O_2 and metal-peroxo species, whereas hypoxic conditions result in a highly reducing cellular environment. The reaction of peroxides with metal centers such as hemes results in formation of oxidants readily capable of oxidizing nitrite to NO_2, which may provide an antipathogenic response as well as a cellular

signal of oxidative stress. Conversely, metal-mediated reduction of nitrite results in the formation of NO, which leads to physiologic processes such as vasodilatation, inhibition of thrombosis, and prevention of leukocyte adhesion to vascular walls. These effects generally provide protection against reperfusion injury, minimizing tissue damage. An intriguing possibility is that nitrite could serve as an ischemic and postischemic conditioning agent because of hypoxia-specific formation of NO. Note that the metal center that yields NO_2 under oxidative conditions will also form NO under reductive conditions. Because formation of the two radicals has different physiologic consequences, the interactions of nitrite with metals may be critical for signaling in biologic systems with a novel level of control based on the redox state of the metal complex. Such chemistry would provide a direct and NOS-independent source of these species, pivotally influencing their formation.

ACIDIFICATION OF NITRITE

Although the recent pharmacologic properties are intriguing, nitrite enjoys a long history of use for medicinal and health remedies. Nitrite possesses antibacterial properties and has been in use as a food preservative for several thousand years (68, 70). The red color of meat was shown to be the result of nitrite reduction by myoglobin (3). In medieval and ancient times, remedies for infections often contained nitrite (68). Studies have shown that nitrite and nitrate in the gastrointestinal tract are part of the antipathogen response and provide another system to combat infection (70, 71).

However, in the 1970s, it was discovered that carcinogenic nitrosamines could be formed from nitrite (56). Studies indicated that the acidic conditions of the stomach could lead to nitrosamine formation, raising the possibility of increased cancer risk (75, 106). The elevated levels of nitrite and nitrate in plasma during infections presented the possibility that nitrosamines could be formed under such conditions. These potential negative implications raised concerns about the safety of nitrite and nitrate. However, in more recent studies, it has been argued that nitrite and nitrate in food do not significantly increase the risk of cancer and provide some protection against bacterial infection (70). Such antibacterial activity of nitrite has been proposed to be due to NO formation from acidified nitrite (68). In 1993, Klebanoff (61) proposed an alternate mechanism that could be important to the antipathogenic properties of nitrite, involving nitrite oxidation to NO_2 via a peroxidase-type mechanism. More recently, acidic nitrite, forming NO_2 and N_2O_3, was shown to have powerful effects on tuberculosis (108). Furthermore, study of NO_2 transport indicates that this species can readily migrate at low concentrations into cells (33).

OXIDATION OF NH_2OH AND NOHA

Although the production of NO from NOS, with subsequent activation of soluble guanylyl cyclase to convert GTP to cGMP, is well established, biosynthesis of NO may not be the sole functionality of NOS. For a free radical, NO is remarkably unreactive and is consumed primarily by association with redox-active metals, oxygen species, and other free radicals (104). Nevertheless, the half-life, and consequently the diffusion distance, of NO are limited (66). Production of more stable nitrogen oxides by NOS may therefore offer the potential to extend the duration, range, and specificity of signaling.

Under inflammatory conditions, catalytic turnover to NO can be interrupted such that NOHA is released at a rate of $\leq$30% (14). In addition, NH_2OH has been detected during turnover of mammalian NOS (90), although the NOS enzymes are not the sole endogenous source of NH_2OH. For instance, despite the inefficiency in iNOS turnover, the elevated levels of NO produced (93) can lead to nitrosative stress conditions. Such altered cellular or tissue status may cause an increase in formation of RSNOs (104), which can decompose to NH_2OH (4, 39, 107). Certainly, RSNOs also decompose by alternative mechanisms, particularly in the presence of proteins such as alcohol dehydrogenase (47), superoxide dismutase (55, 97) and thioredoxin, or fatty acids such as lipoic acid (93). However, the formation of a reduced nitrogen oxide like NH_2OH is intriguing, given the oxidizing environment of mammalian cells.

NOHA can serve as a direct substrate for NOS (14, 15, 46, 63), but is otherwise not known to elicit biologic effects directly. However both NH_2OH and its structural derivative NOHA can be oxidized by two electrons by peroxidases (58) in a Fenton-type reaction (59, 86) (Eq. 1).

$$Fe(V) + H_2O_2 + NOHA/NH_2OH \rightarrow HNO + Fe(III) \quad (1)$$

Analogously, NH_2OH is a known intermediate in the bacterial reduction of nitrite to ammonia via heme proteins. The NH_2OH intermediate is postulated to be preceded by an HNO complex (50).

THE BIOLOGY OF HNO

To date, comparative studies suggest that the physiologic responses to HNO are distinct from those of its redox cousin NO (36, 58, 73). For instance, HNO donors have unique inotropic and lusitropic cardiovascular properties (80). HNO modulates neuronal calcium channel function (22, 37, 57) but does not stimulate release of catecholamines in contrast to NO (44, 45). HNO also is a myocardial preconditioning agent (79) that functions, unlike NO, in an O_2-dependent manner (69). HNO can activate latent transforming growth factor-β (TGF-β), whereas NO does not (67). Additionally, the hexose monophosphate shunt in macrophages is downregulated by NO by activated by NO (21). That HNO and NO have unique biologic activities suggests that mechanisms that generate HNO may be of physiologic importance in the regulation of cellular functions in a fashion complementary to NO. However, several biochemical factors must be considered, particularly the mechanisms by which HNO can be formed, such that it mediates specific biologic properties.

An intriguing possibility for endogenous HNO production is from NOS. Hobbs *et al*. (49) first suggested that HNO was

a possible product of NOS. Furthermore, Schmidt *et al.* (90) showed that N_2O and NH_2OH, both further metabolites of HNO, can be generated from NOS, which was later corroborated by Ishimura *et al.* (54). Furthermore, the addition of high concentrations of superoxide dismutase (SOD) was suggested to oxidize HNO to NO, thus increasing the observable amount of NO (90). Ferrous-nitrosyl heme has been shown to form from tetrahydrobiopterin-depleted NOS, indicating again that HNO is produced under these conditions (2).

The chemical basis for the pharmacologic effects of HNO is commonly considered to result from association with metals or thiols. Binding of HNO to metals results in reductive nitrosylation (Eq. 2). The NO thus formed can either be released or remain associated to the metal, depending on the protein. The reaction of HNO with thiols produces a hydroxylamine derivative RSNHOH (>10^5 M/s) (8, 25, 58) (Eq. 3). The fate of these species (Eqs. 2 and 3) depends on the availability (concentration) and affinity (rate constant) of the reactants.

$$HNO + Fe(III) \rightarrow Fe(II)NO + H^+ \quad (2)$$

$$HNO + RSH \rightarrow RSNHOH \quad (3)$$

The better to understand the biochemistry of these processes and the competition between HNO formation and NO, we have developed a high-performance liquid chromatography (HPLC) method to detect different thiol adducts that specifically represent HNO and NO formation. HNO reacts with GSH to generate GSNHOH (Eq. 3) (107), which either can react with a second GSH to form GSSG and NH_2OH or can isomerize to $GS(O)NH_2$ (Eqs. 4 and 5)

$$GSH + GSNHOH \rightarrow GSSG + NH_2OH \quad (4)$$

$$GSNHOH \rightarrow GS(O)NH_2 \quad (5)$$

We have found that at concentrations <500 μ*M* thiol, $GS(O)NH_2$ is the primary product, and conversely that nitrosation of GSH to yield GSNO occurs via NO formation (Donzelli et al., unpublished data).

In addition to peroxidases, other heme proteins will oxidize NH_2OH to HNO in the presence of H_2O_2. Our preliminary data, using the same HPLC system, indicate that horseradish peroxidase (HRP), myoglobin, and hemoglobin readily generate $GS(O)NH_2$ from NH_2OH and to a lesser degree from NOHA (Fig. 2).

In contrast, catalase produces GSSG rather than $GS(O)NH_2$. From the comparison of the reactivity of HNO with thiol and the heme complexes, some insights can be gained (74). The slowest HNO release was observed with catalase, whereas hemoglobin and myoglobin are somewhat faster. This suggests that HNO cannot escape from the protein pocket to react with the thiols before recombining with the

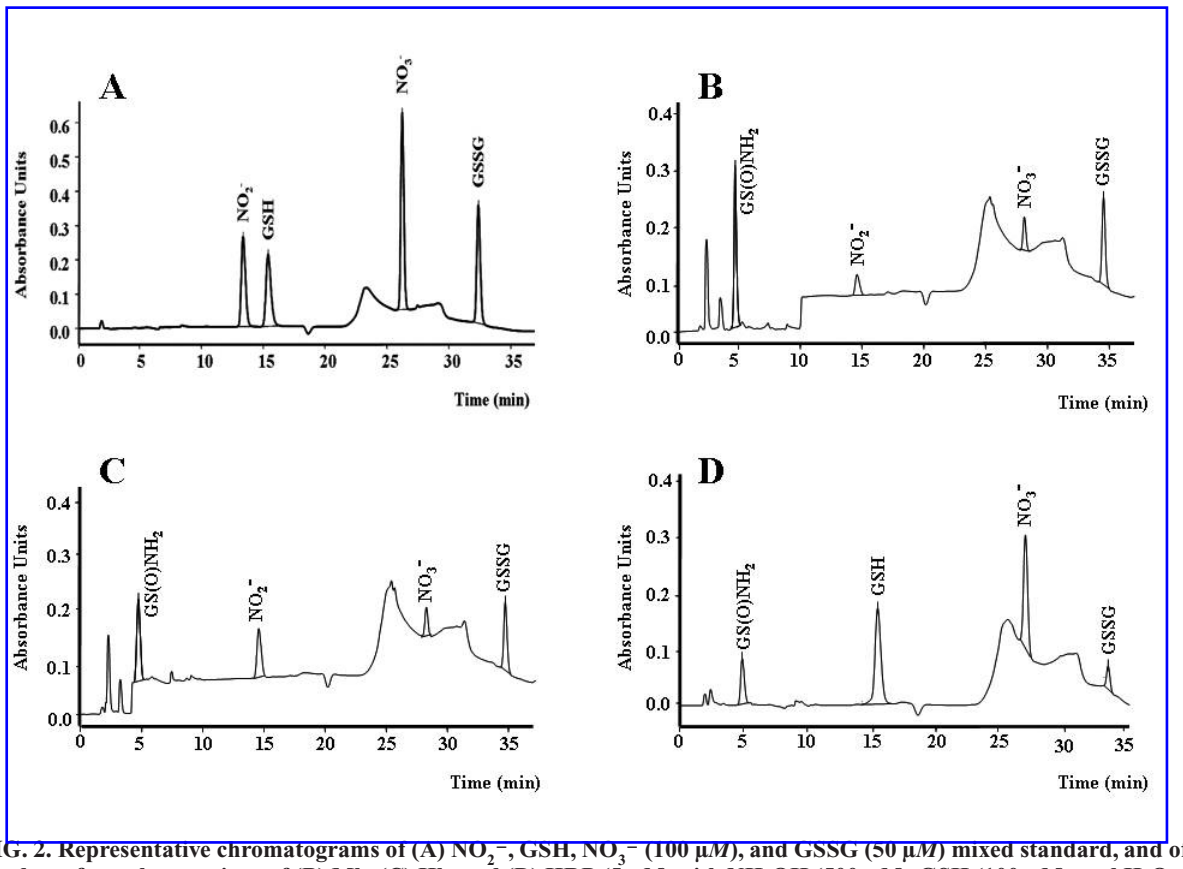

FIG. 2. Representative chromatograms of (A) NO_2^-, GSH, NO_3^- (100 μ*M*), and GSSG (50 μ*M*) mixed standard, and of the products from the reactions of (B) Mb, (C) Hb, and (D) HRP (5 μ*M*) with NH_2OH (500 μ*M*), GSH (100 μ*M*), and H_2O_2 (100 μ*M*) incubated in 10 m*M* phosphate buffer (50 μ*M* DTPA, pH 7.4) for 10 min at 37°C.

ferric heme to form the metal nitrosyl. Thus HNO formation from metal oxidation will be dependent on the rate of escape of the HNO from the protein-active site and on the concentration and reactivity of the molecular target.

One important consequence of HNO formation via metal-mediated oxidation is that the back-reaction to NO prevents HNO from reacting with O_2 to generate intermediates that can damage biomolecules such as DNA (103). This eliminates the potential chemical toxicity of HNO and may protect against oxidative stress mediated by peroxidases or Fenton oxidation. The reaction between HNO and O_2 is 1,000 times slower than the reaction with GSH (103). *In vivo*, the effective concentration of O_2 is 15–40 μ*M*; in cardiac myocytes, myoglobin and GSH concentrations are considerably higher (>1 m*M*). This suggests that HNO formation will not have any chemical toxicity, in contrast to millimolar concentrations of HNO donors (102). Indeed, preliminary data suggest that NOHA abates the cytotoxicity of H_2O_2, indicating that chemistry of this NOS product provides antioxidant capacity.

CONCLUSION: A NEW PERSPECTIVE ON METABOLITES OF NOS

Metal-mediated oxidation of nitrite, NOHA, and NH_2OH offers new biochemical pathways that can generate reactive nitrogen oxides such as NO_2 or HNO under oxidative conditions, which would result in unique cellular signaling (16, 34, 91, 103). Furthermore, under hypoxic conditions, metal complexes can generate NO from nitrite by a reductive mechanism. The ubiquitous nature of these species does not confine these reaction to a specific protein but rather to the location of the metal and the proximity to targets of NO, NO_2, and HNO. This opens numerous possibilities as to the potential for cellular and tissue signaling. These biochemical mechanisms represent an interface between reactive oxygen species and nitrogen oxide signaling in biology. These signaling mechanisms are dependent on the redox status as well as oxygen tension of the cell, providing ideal biochemical reactions to make important sensors available.

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ABBREVIATIONS

$Na_2N_2O_3$, Angeli's salt; GSH, glutathione; GSSG, glutathione disulfide; NH_2OH, hydroxylamine; HRP, horseradish peroxidase; ADH, human alcohol dehydrogenase; NOHA, N^G-hydroxy-L-arginine; NO, nitric oxide; NOS, nitric oxide synthase; RNOS, nitrogen oxide species; RSNO, *S*-nitrosothiols; HNO, nitroxyl; $ONOO^-$; peroxynitrate; $GS(O)NH_2$, sulfonamide; TGF, transforming growth factor.

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Address reprint requests to:
David A. Wink
Radiation Biology Branch
National Cancer Institute/National Institutes of Health
Building 10, Room B3-B35
Bethesda, MD 20892

E-mail: wink@mail.nih.gov

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