The Biological Chemistry of Nitric Oxide as It Pertains to the Extrapulmonary Effects of Inhaled Nitric Oxide

Andrew J. Gow

Department of Pediatrics, Children's Hospital of Philadelphia and the University of Pennsylvania, Philadelphia, Pennsylvania

The chemical properties of nitric oxide (NO) have been studied for over 200 years. However, it is only within the last 20 years that the biological implications of this chemistry have been considered. The classical model of NO action within the vasculature centers on production in the endothelium, diffusion to the smooth muscle, and subsequent activation of guanylate cyclase via binding to its heme iron. In the context of this model, it is difficult to conceptualize extrapulmonary effects of inhaled NO. However, NO possesses complex redox chemistry and is capable of forming a range of nitrogen oxide species and is therefore capable of interacting with a variety of biomolecules. Of particular interest is its reaction with reduced cysteine to form an S-nitrosothiol (SNO). SNOs are formed throughout NO biology and are a post-translational modification that has been shown to regulate many proteins under physiologic conditions. Hemoglobin, which was considered to be solely a consumer of NO, can form SNO in a conformationally dependent manner, which allows for the transport of inhaled NO beyond the realm of the lung. Higher oxides of nitrogen are capable of modifying proteins via nitration of tyrosines, which has been shown to occur under pathologic conditions. By virtue of its redox reactivity, one can appreciate that inhaled NO has a variety of routes by which it can act and that these routes may lead to extrapulmonary effects.

Keywords: lung; nitric oxide; nitrotyrosine; S-nitrosothiol

BIOCHEMICAL HISTORY OF NITRIC OXIDE

The history of chemical experimentation with nitrogen oxides traces back to Priestley's original experiments with the "gaseous oxide of azote" (1). Subsequently, Sir Humphry Davy performed the first biological experiments with NO, termed "nitrous air," while examining the effects of various gases on respiration and the circulatory system (2). Davy noticed that inhaling nitric oxide (NO)–containing gases caused animals "to die infinitely sooner than in common air or oxygen: but not nearly so short a time as in gases incapable of effecting positive changes in the venous blood." Davy noted that tissues from these animals were "purple red" in color, consistent with the formation of nitrosylheme within the periphery. It is fair to say that Humphry Davy, among his many other great achievements, was the first to discover an extrapulmonary effect of NO.

Nearly 200 years later, the Nobel Prize in Physiology or Medicine was awarded for the discovery of the endothelium-derived relaxing factor and its identification as NO (3). The award recognized three seminal experiments: (*1*) the discovery that NO activated guanylate cyclase to induce smooth muscle cell relaxation (4), (*2*) the observation that the endothelium released a diffusible factor that was responsible for acetylcholine-mediated relaxation of blood vessels (5), and (*3*) the identification of the released factor as NO by spectroscopic examination of reaction products with hemoglobin (6). Along with the identification of NO synthase (NOS), these experiments allow one to understand the classical model of NO action in which a stimulus, such as acetylcholine, causes NO production within the endothelium followed by diffusion into the smooth muscle cell, activation of guanylate cyclase, and vasorelaxation. This model underlies much of our understanding of NO within vasculature and the rationale behind the use of NO as an inhaled therapy.

There are a number of lines of evidence indicating that this model may not explain the entirety of NO biology. Indeed, it seems there may be an extrapulmonary role for inhaled NO. The first question relates back to the discoveries of Davy who noted color changes that one might expect from the formation of nitrosylheme. Early practitioners in the field of NO biology found it difficult to envisage how NO produced in the endothelium could diffuse into smooth muscle cells without being readily consumed by its potent reaction with hemoglobin (7). A number of solutions to this dilemma have been proposed since that time, but for the purposes of this article it is sufficient to note that red blood cells are now regarded more as deliverers of NO rather than consumers (8). The second limitation of the classical diffusion model of NO to guanylate cyclase is that oxodiazole quinoaxlin (ODQ), a potent guanylate cyclase inhibitor, does not entirely inhibit NO-mediated vasorelaxation. This indicates that there may be other biochemical pathways for NO (9). Perhaps the most convincing evidence of the complexity of NO biology is its multiplicity of function: NO is active in every major organ system and possesses over 36 different functions, many of which seem to operate independently of guanylate cyclase (10). This pluripotency, by a wide range of mechanisms, points to a greater range of NO chemistry than simple diffusion and hemoprotein binding.

A final observation left unexplained by the classical diffusion model of NO is the apparent regionalization of function by different NOS isozymes. For instance, the localization of endothelial NOS (eNOS) within caveolae is critical not only to its activity but also in controlling its targets (11). Furthermore, it has been demonstrated that, within a single cardiac myocyte, different NOS isozymes can affect different targets. Neuronal NOS (nNOS) within the myocyte's sarcoplasmic reticulum targets the ryanodine receptor to increase the rate of intracellular calcium release and thus aid in cardiac contractility. However, eNOS in the sarcolemma seems to negatively affect calcium entry via the L-type calcium channel, whereas a mitochondrially located NOS is critical in controlling respiration (12). Perhaps the most remarkable thing about these different NOS enzymes within the one cell is that it seems that their functions do not overlap (i.e., the NO made by each NOS seems to be localized its area of production).

(*Received in original form June 14, 2005; accepted in final form September 7, 2005*)

Supported by National Institutes of Health grant HL074155.

Correspondence and requests for reprints should be addressed to Andrew J. Gow, Ph.D., Children's Hospital of Philadelphia, University of Pennsylvania Abramson Research Center, 3517 Civic Center Boulevard, Philadelphia, PA 19104. E-mail: gow@email.chop.edu

Proc Am Thorac Soc Vol 3. pp 150–152, 2006
DOI: 10.1513/pats.200506-058BG
Internet address: www.atsjournals.org

REDOX CHEMISTRY OF NO

To understand the complex biology of NO, it is necessary to consider the redox chemistry of nitrogen oxides. As Priestley (1) and Davy (2) discovered in their experiments, nitrogen oxides are readily converted into other redox forms of nitrogen in the presence of simple oxidants and/or reductants. Perhaps the easiest way to conceptualize this is to think of a redox spectrum for NO (Figure 1) (10). An analogous spectrum for partially reduced oxygen species, such as superoxide, hydrogen peroxide, and the hydroxyl radical, is easy to imagine. These reduced oxygen species exist in a spectrum that extends from fully oxidized (e.g., molecular oxygen) to fully reduced (e.g., water). A similar but more complex spectrum can be envisaged for nitrogen running from nitrate, fully oxidized, to ammonia, fully reduced. In between is a wide range of partially reduced forms of nitrogen, many of which are oxides, that are sometimes referred to as reactive nitrogen and oxygen species. One of the key features of this spectrum is the arc on which it is drawn. Any species that exists on either slope of the arc is far more readily converted further downhill than it is in the other direction. In other words, dinitrogen tetroxide is readily converted to nitrate but requires a large energetic input to be converted to dinitrogen trioxide. Species at the top of the arc are readily oxidized or reduced; this is especially true for nitrogen monoxide, which is easily converted into nitrosonium or nitroxyl equivalents. Therefore, when produced in the presence of appropriate reactive targets, NO can be readily converted into other nitrogen oxide moieties, each of which may have distinct biochemical properties.

The concept of "reactive exposure" was introduced to the NO field by Beckman (13) with his consideration of the formation of peroxynitrite from the reaction of NO with superoxide. According to this principle, the relative amount of a compound (e.g., NO) that reacts with its respective targets is determined by the relative concentrations of those targets and the reaction rates of the compound with each of them. For example, the reaction of NO with superoxide is high ($\sim 10^{10}$ μM/s), but the physiologic concentration is low ($\sim$ 0.1–1 nM) (13). On the other hand, the reaction of NO with a metal center is lower ($\sim 10^{7}$ μM/s), but the reactant concentration is much higher (~ 10 μM). This makes the reactive exposure for metal centers tenfold higher than that for superoxide (10^{8} vs. 10^{7}). If we extend this reasoning and include reaction with thiols and molecular oxygen, we see a reactive exposure of approximately 10 (8). These numbers are estimates, and the local concentrations of the reactive targets vary greatly within the subcompartments of biological systems.

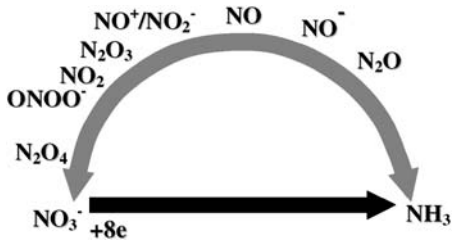

Figure 1. A redox spectrum for nitrogen. Some of the various oxides of nitrogen are portrayed on a redox arc extending from fully oxidized (nitrate) to fully reduced (ammonia). Full reduction of nitrate to ammonia requires the addition of eight electrons. The positioning of nitric oxide, nitrosonium, and nitroxyl at the top of the arc indicates that these species are relatively easily oxidized or reduced. The presence of the higher oxides (e.g., dinitrogen tetroxide) on the left of the arc indicates that reduction of these species is relatively difficult and that they are more easily converted to fully oxidized nitrate.

However, under physiologic conditions, it is predicted that NO's reactions with metal centers, thiols, and oxygen will predominate. The preponderant products of this lower oxide chemistry of NO, therefore, would be metal-nitrosyls, S-nitrosothiols (SNOs), and nitrite. However, because the reaction rate for NO with superoxide is so high, a small increase in the concentration of this reactant results in a large increase in reactive exposure (a 1-nM increase would lead to a 10-fold increase in reactive exposure). Therefore, even under a mild oxidative stress, one would predict that higher oxide chemistry of NO (i.e., the nitration of tyrosine, lipids, DNA, and the formation of nitrate) would occur. We have shown that even the minor inflammatory lung disease that occurs in surfactant protein D knockout mice results in a major shift from lower to higher oxide chemistry within the tissue and in the lung lining (14).

S-Nitrosothiols

One of the products of lower oxide chemistry of NO is S-nitrosothiol (SNO). SNO was first suggested to have a role within the vascular biology of NO in 1993 by Stamler and colleagues (15). Originally, not much attention was given to the possibility of SNOs playing a major role in the physiology of NO because it was believed they could be formed only via autooxidation of NO (16), a kinetically unfavorable process under physiologic conditions. Since that time, it has become evident that SNOs can be formed by the direct interaction of NO with thiols coupled with electron abstraction (17). This reaction can proceed through a radical intermediate, as has been seen in p21ras (18), and can be catalyzed by metals such as copper (19). Two metalloproteins, hemoglobin and ceruloplasmin, have been shown to catalyze the formation of SNO (20, 21). Although SNO formation from NO and SH is a nominal one-electron oxidation, this oxidation can occur in three ways: (*1*) conversion of NO to nitrosonuim cation, (*2*) thiol oxidation to a thiyl radical, (*3*) or electron abstraction after formation of a RSNOH radical intermediate.

We have shown that an antibody to the SNO moiety demonstrated significant specificity toward SNO and that it could be used in immunohistochemistry (22). The antibody enabled the identification of SNO formation from each of the isoforms of NOS. Using mouse thoracic aorta to study eNOS, we showed that there was minimal SNO formation in a precontracted aorta but that acetylcholine stimulated SNO formation in the endothelium and the smooth muscle in a manner inhibitable by the NOS inhibitor L-NAME. To model nNOS activity, we examined PC-12 cells, which can be induced to express nNOS without cell differentiation by low levels of nerve growth factor, but no SNO in unstimulated cells. Our studies showed significant SNO production in cells treated with nerve growth factor. Finally, we used cultured RAW 264.7 cells, an established macrophage model, to demonstrate that these cells induced iNOS and SNO formation when incubated with cytokines. Therefore, it seems that SNO production is a potentially universal feature of NOS activity.

Hemoglobin reveals an important aspect of the S-nitrosylation of proteins (i.e., that there is a structural component to their formation and stability). Hemoglobin contains a cysteine residue at position 93 on the β chain that is conserved throughout mammals and birds and is present in many fish. This cysteine's reactivity has long been known to be altered by the conformational state of the hemoglobin (23). In the R (relaxed, oxygenated) structure, the cysteine faces inward toward the heme moiety and is positioned between a histidine and an aspartate residue. However, in the T (tense, deoxygenated) structure, this cysteine is rotated outward toward the salt bridge and away from the heme. In this structure, the positioning between the histidine and aspartate residues is lost. From original work on

the *N*-methyl-D-aspartate receptor, it has been proposed that the flanking of a cysteine by a basic and an acidic residue makes it susceptible to SNO formation (24). One would predict that SNO would form more easily and be more stable in R than in T structure. This was found to be the case in the original studies on SNO hemoglobin by Jia and colleagues (25). Further studies by our group have shown that the conformational change from T to R structure can induce the conversion of iron-nitrosyl hemoglobin to SNO-hemoglobin with the release of an electron (20, 26). NO's reactions with hemoglobin can be classified in one of three ways: (*1*) conservation, by the reversible binding of a NO group to the heme iron; (*2*) consumption, by the irreversible oxidation of NO to nitrate after reaction with bound oxygen; and (*3*) delivery, by the formation of SNO hemoglobin from which NO equivalents are released upon conversion to the T state (27). The last of these functions are discussed elsewhere in this issue.

NITROTYROSINE

Higher concentrations of oxidative species promote the conversion of NO to higher oxide forms, such as nitrogen dioxide and peroxynitrite. One result of the production of such species is the formation of nitrotyrosine (28). The production of these species may have signaling consequences of their own as well as reducing the quantity of NO available for lower oxide reactions. In a model of hyperoxic lung injury, we showed that nitrotyrosine formation was coupled with a reduction in SNO production. Furthermore, the introduction of inhaled NO resulted in increased production of SNO without altering nitrotyrosine formation (29). We made similar observations in a human disease with an inflammatory component (bronchopulmonary dysplasia). By immunohistochemistry, we were able to show that there was extensive formation of SNO and nitrotyrosine, whereas normal-term lung showed only SNO (22).

CONCLUSIONS

The chemistry of NO within biological systems is more complex than diffusion and binding. This diverse chemistry may allow NO to escape inactivation within the lung and may allow the production of extrapulmonary effects of inhaled NO. In this article, I have discussed two other such reactivities for NO, S-nitrosylation and nitration. These two post-translational modifications have considerable effect on protein function, although in the case of nitration the effect is usually a loss of function rather than a regulation. SNO formation bears all the hallmarks of a post-translational modification with signaling consequences. Its formation is controlled by target protein structure (24) and can be catalyzed by known proteins (20, 21), while its removal is easily achieved and can also be catalyzed by known proteins (30). Perhaps most important, nitrosylation of cysteine residues can result in protein activation or inhibition depending upon the target. It seems that alternate redox activities of NO may supply the chemical basis for extrapulmonary effects of NO.

Conflict of Interest Statement: A.J.G. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

References

1. Priestley J. Experiments and observations on different kinds of air, 2nd ed. London: J. Johnson; 1775.
2. Davy H. The collected works of Sir Humphry Davy. London: Continuum International Publishing; 1839.
3. Hansson G, Jornvall GK, Lindahl SGE, Ringertz N. The Nobel prize in physiology or medicine 1998. Stockholm, Sweden: The Nobel Committee for Physiology or Medicine at the Karolinska Institute; 1998
4. Arnold WP, Mittal CK, Katsuki S, Murad F. Nitric oxide activates guanylate cyclase and increases guanosine 3′:5′-cyclic monophosphate levels in various tissue preparations. *Proc Natl Acad Sci USA* 1977; 74:3203–3207.
5. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 1980; 288:373–376.
6. Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc Natl Acad Sci USA* 1987;84:9265–9269.
7. Lancaster JR Jr. Simulation of the diffusion and reaction of endogenously produced nitric oxide. *Proc Natl Acad Sci USA* 1994;91:8137–8141.
8. Gow AJ. Nitric oxide, hemoglobin, and hypoxic vasodilation. *Am J Respir Cell Mol Biol* 2005;32:479–482.
9. Homer KL, Wanstall JC. Cyclic GMP-independent relaxation of rat pulmonary artery by spermine NONOate, a diazeniumdiolate nitric oxide donor. *Br J Pharmacol* 2000;131:673–682.
10. Gow AJ, Ischiropoulos H. Nitric oxide chemistry and cellular signaling. *J Cell Physiol* 2001;187:277–282.
11. Segal SS, Brett SE, Sessa WC. Codistribution of NOS and caveolin throughout peripheral vasculature and skeletal muscle of hamsters. *Am J Physiol* 1999;277:H1167–H1177.
12. Hare JM, Stamler JS. NOS: modulator, not mediator of cardiac performance. *Nat Med* 1999;5:273–274.
13. Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol* 1996;271:C1424–C1437.
14. Atochina EN, Beers MF, Hawgood S, Poulain F, Davis C, Fusaro T, Gow AJ. Surfactant protein-D, a mediator of innate lung immunity, alters the products of nitric oxide metabolism. *Am J Respir Cell Mol Biol* 2004;30:271–279.
15. Stamler JS, Jaraki O, Osborne J, Simon DI, Keaney J, Vita J, Singel D, Valeri CR, Loscalzo J. Nitric oxide circulates in mammalian plasma primarily as an S-nitroso adduct of serum albumin. *Proc Natl Acad Sci USA* 1992;89:7674–7677.
16. Kharitonov VG, Sundquist AR, Sharma VS. Kinetics of nitrosation of thiols by nitric oxide in the presence of oxygen. *J Biol Chem* 1995; 270:28158–28164.
17. Gow AJ, Buerk DG, Ischiropoulos H. A novel reaction mechanism for the formation of S-nitrosothiol in vivo. *J Biol Chem* 1997;272:2841–2845.
18. Williams JG, Pappu K, Campbell SL. Structural and biochemical studies of p21Ras S-nitrosylation and nitric oxide-mediated guanine nucleotide exchange. *Proc Natl Acad Sci USA* 2003;100:6376–6381.
19. English AM, Wilcox DE. Effects of metal ions on the oxidation and nitrosation of cysteine residues in proteins and enzymes. *Met Ions Biol Syst* 2001;38:313–350.
20. Gow AJ, Stamler JS. Reactions between nitric oxide and haemoglobin under physiological conditions. *Nature* 1998;391:169–173.
21. Inoue K, Akaike T, Miyamoto Y, Okamoto T, Sawa T, Otagiri M, Suzuki S, Yoshimura T, Maeda H. Nitrosothiol formation catalyzed by ceruloplasmin: implication for cytoprotective mechanism in vivo. *J Biol Chem* 1999;274:27069–27075.
22. Gow AJ, Chen Q, Hess DT, Day BJ, Ischiropoulos H, Stamler JS. Basal and stimulated protein S-nitrosylation in multiple cell types and tissues. *J Biol Chem* 2002;277:9637–9640.
23. Taylor JF, Antonini E, Brunori M, Wyman J. Studies on human hemoglobin treated with various sulfhydryl reagents. *J Biol Chem* 1966;241: 241–248.
24. Stamler JS, Toone EJ, Lipton SA, Sucher NJ. (S)NO signals: translocation, regulation, and a consensus motif. *Neuron* 1997;18:691–696.
25. Jia L, Bonaventura C, Bonaventura J, Stamler JS. S-nitrosohaemoglobin: a dynamic activity of blood involved in vascular control. *Nature* 1996; 380:221–226.
26. McMahon TJ, Moon RE, Luschinger BP, Carraway MS, Stone AE, Stolp BW, Gow AJ, Pawloski JR, Watke P, Singel DJ, *et al.* Nitric oxide in the human respiratory cycle. *Nat Med* 2002;8:711–717.
27. Frehm EJ, Bonaventura J, Gow AJ. S-Nitrosohemoglobin: an allosteric mediator of NO group function in mammalian vasculature. *Free Radic Biol Med* 2004;37:442–453.
28. Ischiropoulos H. Biological tyrosine nitration: a pathophysiological function of nitric oxide and reactive oxygen species. *Arch Biochem Biophys* 1998;356:1–11.
29. Lorch SA, Foust R III, Gow A, Arkovitz M, Salzman AL, Szabo C, Vayert B, Geffard M, Ischiropoulos H. Immunohistochemical localization of protein 3-nitrotyrosine and S-nitrosocysteine in a murine model of inhaled nitric oxide therapy. *Pediatr Res* 2000;47:798–805.
30. Liu L, Hausladen A, Zeng M, Que L, Heitman J, Stamler JS. A metabolic enzyme for S-nitrosothiol conserved from bacteria to humans. *Nature* 2001;410:490–494.