Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 10345–10347, September 1998

Commentary

Nitric oxide in plant immunity

Alfred Hausladen and Jonathan S. Stamler*†‡*

†*Howard Hughes Medical Institute and *Departments of Medicine and Cell Biology, Duke University Medical Center*

Once touted as a toxin and then as a powerful effector of cardiac, brain, lung, genitourinary, gastrointestinal, and immune function—but ultimately exposed by the relatively unremarkable phenotypes of transgenic mice deficient in each of the three major NO synthase (NOS) isoforms—NO and molecules derived from it are now revealing more subtle, but highly influential, roles in signaling. So it is that more than one decade since nitric oxide biosynthesis was discovered in animals, scientists are only beginning to unravel the major function of this complex system (Fig. 1). Ironically, the very reactions of NO with redox centers in proteins and membranes, that were originally identified with injurious and polluting effects of the molecule, are now being established as molecular components of signal transduction pathways controlling smooth muscle tone, cell proliferation and adhesion, platelet activation, force production in heart and skeletal muscle, respiration, neurotransmission, hormone secretion, ion channel activity, apoptosis, transcriptional mechanisms, and host responses to infection (1–5). That NO has been widely adapted to serve a signaling role in biology is underscored by the distribution of NOSs throughout the animal kingdom (6) and in some fungi and bacteria (7–9).

Production of NO is not confined to organisms containing NOS. Rather, nitrate reduction by bacteria, fungi, and plants is known to be an alternative source (10–13). That is, NO is a byproduct of denitrification, nitrate assimilation, or respiration. Plants even might be exposed to NO produced from soil microorganisms. But if NO's larger role in signaling is only just being appreciated in mammals in which regulated enzymatic production has been demonstrated clearly in virtually every cell, then imagine how distant a notion this is in plants in which NO can aggravate ozone-induced injury on the one hand (14) and regulation of NO biosynthesis and of physiological functions has not been shown, on the other hand. Exciting new evidence now promises to challenge this common view. Recent studies suggest that plants contain a NOS-like enzyme (a deliberate means for producing NO-related activity from substrate L-arginine) (9, 15) and implicate NO in plant growth and development, signal transduction, and disease resistance (16–18). In a previous issue, on page 10328 of the *Proceedings*, Durner *et al.* (19) take the case for NO regulation of vital plant functions a significant step further. They show that "NOS" protects tobacco plants from viral infection by triggering the induction of defense-related genes. Remarkably, NO does so by using the same signal transduction pathways that it uses in mammals.

Plants employ many strategies to defend themselves from predators and pathogens (20). One mechanism of self-defense is particularly reminiscent of our own innate immune response. On recognition of pathogens, plant cells produce reactive chemicals and signaling molecules, some of which may initiate death programs to limit the spread of the infection. This rapid (or "hypersensitive") response is followed by the acquisition of resistance to a range of pathogens at sites distal to the original infection (termed "systemic-acquired resistance") (21–23). Otherwise translated, chemical signals produced at the site of infection travel to distant sites, and there they convey a message that leads to induction of "pathogenesis-related" (PR) defense proteins. Most prominent among the signaling candidates are salicylic acid and H_2O_2 (21), but others are the subjects of intensive research. Durner *et al.* (19) now demonstrate that plant NOS and NO-related molecules increase levels of salicylic acid and PR protein. Thus, NOS may be a protective locus in plants, and its product NO, one of the chemicals active in plant defense. Additional biochemical and genetic studies of plant NOS are needed, however, for proof-of-principle. Indeed, plant NOS has not yet been purified, a cDNA encoding the protein has not been isolated and alternative sources of L-arginine metabolism (to citrulline) have not been excluded.

NO signals in biology are typically labeled as either dependent on cGMP or independent thereof—a tribute to the seminal work of Murad and coworkers (24) who discovered that NO activates guanylate cyclase, and also a reflection of the breadth of cGMP effects (25). However, such an assignment presupposes that the downstream targets of cGMP and the alternative cGMP independent "pathway"—involving nitrosylation of proteins (3)—are known and that the signaling components of these pathways are different (Fig. 1). It also presupposes that the tools used to differentiate between the signaling pathways or to elucidate NO's mechanism of action are specific to one pathway or the other. In point of fact, the targets of NO/cGMP are, with rarest of exception, not known. Thus, the possibility that the target of cGMP-dependent protein kinase, for example, might lie upstream in a signaling cascade—which also is regulated distally by nitrosylation—or downstream of an alternatively regulated target, is not usually considered. Indeed, only very recent work in transgenic mice unequivocally establishes cGMP-dependent protein kinase as the transducer of the cGMP component (26, 27) of the NOS signal that regulates vascular smooth muscle tone (28). How cGMP-dependent protein kinase regulates various smooth muscle and other functions is still very much an open question.

Interpretation of the mechanism of NO action is made more difficult by the limited specificity of pharmacological agents used to inhibit guanylate cyclase and the problems inherent to measurements of nitrosylated proteins in cells. In particular, guanylate cyclase inhibitors such as methylene blue (and probably LY83583; refs. 29 and 30) oxidize thiols (31) and transition metals—i.e., NOs major targets—and may inhibit NOS more potently than guanylate cyclase (32). These molecules also may generate superoxide or otherwise modify NO action (29, 30, 33). (Thus, one must be very cautious of interpretations based on use of LY83583; ref. 19). A new generation of guanylate cyclase inhibitors, led by ODQ (1 H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one), shows more promise (27), but the notion that they are specific seems

Abbreviation: NOS, NO synthase.

The companion to this commentary begins on page 10328 in issue 17 of volume 95.

‡To whom reprint requests should be addressed. e-mail: STAML001@mc.duke.edu.

© 1998 by The National Academy of Sciences 0027-8424/98/9510345-3$2.00/0
PNAS is available online at www.pnas.org.

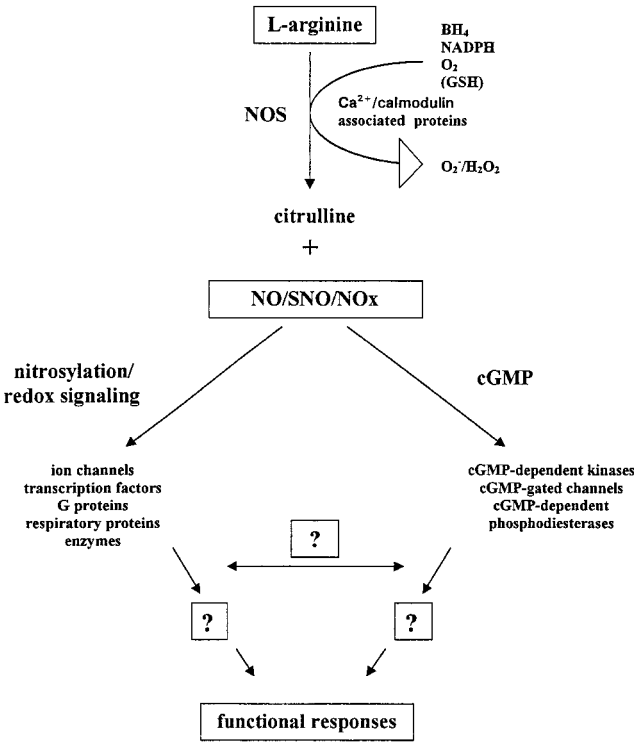

FIG. 1. The NO-signaling pathway. NOSs convert L-arginine to citrulline and a family of NO-related molecules. Exact N-oxide yields and identities vary as a function of experimental condition. The reaction requires cosubstrates/cofactors NADPH, O_2, and tetrahydrobiopterin (BH_4). Glutathione (GSH) may sustain enzyme activity and/or influence reaction product. Superoxide/hydrogen peroxide (O_2^-/H_2O_2) are typically generated by NOS, at least to some degree. NOSs may be regulated by Ca^{2+}/calmodulin and other associated proteins. NO-elicited signals are mediated by cGMP, nitrosylation, or both. Although a growing list of signaling pathways are influenced by NO, sites of regulation by NO and NO-dependent interactions between signal transduction cascades are as yet poorly characterized. SNO, S-nitrosothiol; NOx, other oxides of nitrogen, in this case perhaps also including low mass metal-nitrosyl compounds.

incongruous with their mechanism of action. They appear to oxidize transition metals or at least heme iron (27). Inasmuch as metals are found in many NO targets and also are probably required to further S-nitrosylation of signaling elements (2, 34), such agents may be used to exclude, but not to selectively implicate, the cGMP pathway. Moreover, the heterocyclic nature of ODQ raises the concern that it may block ion channels. It is not surprising that reports on lack of ODQ specificity have appeared recently in the literature (35, 36).

Durner *et al.* (19) nevertheless have overcome the limitations of pharmacological agents in providing one of the more remarkable examples of NO/cGMP-dependent signaling and transcriptional regulation in a cellular control mechanism. Not only is the case well made—they measure intracellular rises in cGMP that precede functional responses and reproduce NOS effects with cGMP analogues and NO donors—but it also provides new insights into the temporal aspect of the cGMP signal. That said, their discovery is instructive of how embarrassingly little we understand of this classical pathway. Specifically, Durner *et al.* (19) show that NOS activity, stimulated by virus, is followed by a transient intracellular rise in cGMP that long precedes the expression of mRNA-encoding resistance proteins. This cGMP transient and delay in gene transcription is mimicked by application of NO donors with relatively long half-lives. One can only speculate on why cGMP levels fall in the presence of NO or how this signal activates genes in delayed fashion. One might also ask how cGMP coordinates the induction of two defense-minded genes, one of which is dependent on salicylic acid (PR-1) and one of which (phenylalanine ammonia lyase; PAL) is not. And one might also wonder how much cGMP regulation in both plants and animals has been missed by not considering the nature of the transient that may dictate the response.

Durner *et al.* (19) tentatively assign a NO resistance role to cyclic ADP ribose, which in sea urchin (37), plants (38, 39), and some mammalian cells (40) can release calcium from ryanodine receptor-like internal stores, and also identify a component of the NO signal, which is independent of cGMP. It may turn out to be important that they find plant defense genes are induced by cyclic ADP ribose (19). However, there are caveats with these data, which do not help to clarify the generally confusing relationship between cyclic ADP ribose, ryanodine receptor activity, and cGMP or make a case for a cause-and-effect relationship between NOS activity and cyclic ADP ribose action. On the other hand, there is strong evidence that ryanodine receptor-elicited calcium release, at least in skeletal muscle and cardiac muscle cells, is regulated by S-nitrosylation/oxidation of the channel (41–43). This result might well explain the cGMP-independent effect of NO, particularly given that calcium release from internal stores induced the expression of plant defense genes (19). More importantly, these data emphasize the unusual complexity of the cellular response to NO, which may be mediated by phosphorylation-, nitrosylation- or calcium-controlled mechanisms. How these pathways interconnect and when and where they operate independently are areas of active study.

What additional roles might be predicted for NO and related molecules in plant resistance? Features of self defense by plants are similar enough to those of mammals (20, 21, 44) to believe that plants might likewise exploit the (hypersensitive) response to increase NO reactivity toward microbes. For example, NO may be converted by the respiratory burst oxidase into a bactericidal agent as a consequence of its reaction with superoxide (45). Alternative reactions with thiols also can enhance NO potency (46). Intriguingly, there is even a suggestion that NO-related molecules subserve such an antimicrobial function in plants. The clue comes from observations in bacteria that possess an inducible flavohemoglobin, which has just been shown to provide protection from nitrosative stress (47). That is, the protein metabolizes NO and S-nitrosothiols (SNO) (A.H. and J.S.S., unpublished results). The corresponding flavohemoglobin gene in the plant pathogen Erwinia is required for virulence (48), raising not only the possibility that NO/SNO are indeed used in bacterial killing by plants, but also that plant pathogens have evolved sophisticated resistance mechanisms to counter a nitrosative threat.

1. Schmidt, H. H. & Walter, U. (1994) *Cell* **78,** 919–925.
2. Stamler, J. S. (1994) *Cell* **78,** 931–936.
3. Stamler, J. S., Toone, E. J., Lipton, S. A. & Sucher, N. J. (1997) *Neuron* **18,** 691–696.
4. Nathan, C. & Xie, Q. W. (1994) *Cell* **78,** 915–918.
5. Nathan, C. (1995) *Cell* **82,** 873–876.
6. Ottaviani, E., Franchini, A. & Franceschi, C. (1997) *Int. Rev. Cytol.* **170,** 79–141.
7. Morita, H., Yoshikawa, H., Sakata, R., Nagata, Y. & Tanaka, H. (1997) *J. Bacteriol.* **179,** 7812–7815.
8. Chen, Y. & Rosazza, J. P. N. (1995) *J. Bacteriol.* **177,** 5122–5128.
9. Ninnemann, H. & Maier, J. (1996) *Photochem. Photobiol.* **64,** 393–398.
10. Zumft, W. G. (1997) *Microbiol. Mol. Biol. Rev.* **61,** 533–616.
11. Ji, X.-B. & Hollocher, T. C. (1989) *Biochem. Arch.* **5,** 61–66.
12. Nakahara, K., Tanimoto, T., Hatano, K., Usuda, K. & Shoun, H. (1993) *J. Biol. Chem.* **268,** 8350–8355.
13. Dean, J. V. & Harper, J. E. (1988) *Plant Physiol.* **88,** 389–395.
14. Neighbor, E. A., Pearson, M. & Mehlhorn, H. (1990) *Atmos. Environ.* **24,** 711–715.

15. Cueto, M., Hernandez-Perera, O., Martin, R., Bentura, M. L., Rodrigo, J., Lamas, S. & Golvano, M. P. (1996) *FEBS Lett.* **398,** 159–164.
16. Leshem, Y. Y. & Haramaty, E. (1996) *J. Plant Physiol.* **148,** 258–263.
17. Pfeiffer, S., Janistyn, B., Jessner, G., Pichorner, H. & Ebermann, R. (1994) *Phytochemistry* **36,** 259–262.
18. Noritake, T., Kawakita, K. & Doke, N. (1996) *Plant Cell Physiol.* **37,** 113–116.
19. Durner, J., Wendehenne, D. & Klessig, D. F. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 10328–10333.
20. Ryan, C. A. & Jagendorf, A. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 4075.
21. Hammond-Kosack, K. E. & Jones, J. D. (1996) *Plant Cell* **8,** 1773–1791.
22. Ryals, J. A., Neuenschwander, U. H., Willitis, M. G., Molina, A., Steiner, H. Y. & Hunt, M. D. (1996) *Plant Cell* **8,** 1809–1819.
23. Alvarez, M. E., Pennell, R. I., Meijer, P. J., Ishikawa, A., Dixon, R. A. & Lamb, C. (1998) *Cell* **92,** 773–784.
24. Arnold, W. P., Mittal, C. K., Katsuki, S. & Murad, F. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 3203–3207.
25. Schmidt, H. H., Lohmann, S. M. & Walter, U. (1993) *Biochim. Biophys. Acta* **1178,** 153–175.
26. Bolotina, V. M., Najibi, S., Palacino, J. J., Pagano, P. J. & Cohen, R. A. (1994) *Nature (London)* **368,** 850–853.
27. Brunner, F., Schmidt, K., Nielsen, E. B. & Mayer, B. (1996) *J. Pharmacol. Exp. Ther.* **277,** 48–53.
28. Pfeifer, A., Klatt, P., Massberg, S., Ny, L., Sausbier, M., Hirneiss, C., Wang, G. X., Korth, M., Aszodi, A., Andersson, K. E., *et al.* (1998) *EMBO J.* **17,** 3045–3051.
29. Schmidt, M. J., Sawyer, B. D., Truex, L. L., Marshall, W. S. & Fleisch, J. H. (1985) *J. Pharmacol. Exp. Ther.* **232,** 764–769.
30. Mulsch, A., Busse, R., Liebau, S. & Forstermann, U. (1988) *J. Pharmacol. Exp. Ther.* **247,** 283–288.
31. Jocelyn, P. C. (1972) in *Biochemistry of the SH group* (Academic, London), pp. 104–105.
32. Mayer, B., Brunner, F. & Schmidt, K. (1993) *Biochem. Pharmacol.* **45,** 367–374.
33. Marczin, N., Ryan, U. S. & Catravas, J. D. (1992) *J. Pharmacol. Exp. Ther.* **263,** 170–179.
34. Gow, A. J. & Stamler, J. S. (1998) *Nature (London)* **391,** 169–173.
35. Muller, B., Kleschyov, A. L., Malblanc, S. & Stoclet, J. C. (1998) *Br. J. Pharmacol.* **123,** 1221–1229.
36. Mannick, J. B., Miao, X. Q. & Stamler, J. S. (1997) *J. Biol. Chem.* **272,** 24125–24128.
37. Willmott, N., Sethi, J. K., Walseth, T. F., Lee, H. C., White, A. M. & Galione, A. (1996) *J. Biol. Chem.* **271,** 3699–3705.
38. Wu, Y., Kuzma, J., Marechal, E., Graeff, R., Lee, H. C., Foster, R. & Chua, N. H. (1997) *Science* **278,** 2126–2130.
39. Allen, G. J., Muir, S. R. & Sanders, D. (1995) *Science* **268,** 735–737.
40. Lee, H. C. (1997) *Physiol. Rev.* **77,** 1133–1164.
41. Zahradnikova, A., Minarovic, I., Venema, R. C. & Meszaros, L. G. (1997) *Cell Calcium* **22,** 447–454.
42. Xu, L., Eu, J. P., Meissner, G. & Stamler, J. S. (1998) *Science* **279,** 234–237.
43. Stoyanovsky, D., Murphy, T., Anno, P. R., Kim, Y. M. & Salama, G. (1997) *Cell Calcium* **21,** 19–29.
44. Torres, M. A., Onouchi, H., Hamada, S., Machida, C., Hammond-Kosack, K. E. & Jones, J. D. (1998) *Plant J.* **14,** 365–370.
45. De Groote, M. A., Ochsner, U. A., Shiloh, M. U., Nathan, C., McCord, J. M., Dinauer, M. C., Libby, S. J., Vazquez-Torres, A., Xu, Y. & Fang, F. C. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 13997–14001.
46. Fang, F. C. (1997) *J. Clin. Invest.* **99,** 2818–2825.
47. Crawford, M. J. & Goldberg, D. E. (1998) *J. Biol. Chem.* **273,** 12543–12547.
48. Favey, S., Labesse, G., Vouille, V. & Boccara, M. (1995) *Microbiology* **141,** 863–871.