NITRIC OXIDE AS A DEFENSE MOLECULE OF THE BODY

Hun-Taeg Chung, M.D., Ph.D.

Department of Microbiology and Immunology,
Wonkwang University School of Medicine, and
Medicinal Resources Research Center of
Wonkwang University

I. Mononuclear Phagocytes are the First and Lost Lines of Defense

The immune system is a defense mechanism that operates in vertebrates and has evolved to provide highly adaptive protection against infectious agents. The vertebrate immune system is characterized by a learned self-nonself discrimination mechanism that distinguishes this system from all other protective mechanisms. Recognition of the invading pathogens includes a determination of whether the parasite is essentially extracellular or intracellular, and this in tern leads to the selective activation of one of two classes of effector function: helper T-cells for intracellular parasites and B-cell immunoglobulin "handles" for extracellular parasites. Theses specific recognitive structures of the immune system do not themselves destroy parasites; they do, however, serve to markedly enhance the action of phagocytic cells that kill infectious agents (Fig. 1).

The mononuclear phagocyte is important because it represents both the beginning and the end of the celluar basis of the immune system. It is the beginning of the cellular immune response in the sense that any foreign substance entering the extracellular spaces of the body is first detected as foreign by the mononuclear phagocytes; if antigen enters via blood, the fixed phagocytic cells lining liver sinuses (Kupffer cells) and those to a lesser extent in spleen are able to bind the antigen, and in most cases the subsequent phagocytosis of antigen results in removal of the antigen; any antigen remaining on the outside of these cells, or antigen that migrates elsewhere, is likely to induce an immune response. Pathogenic viral and bacterial antigens are usually able to avoid elimination during the first innate immune round of phagocytosis and establish a focus of proliferating antigen; this is the start of a race between the pathogen and the immune system. Antigen that enters the lung as an aerosol is taken up by mononuclear phagocytes that line the alveolar surfaces. Antigen that enters via a skin is dealt with by a group of mononuclear phagocytes that are termed "Langerhans cells". In brain, usually considered to be an immunologically privileged site inasmuch as cells and even immunoglobulins do not pass the blood-brain barrier, antigen is taken up by cells termed "microglia" which belong to the

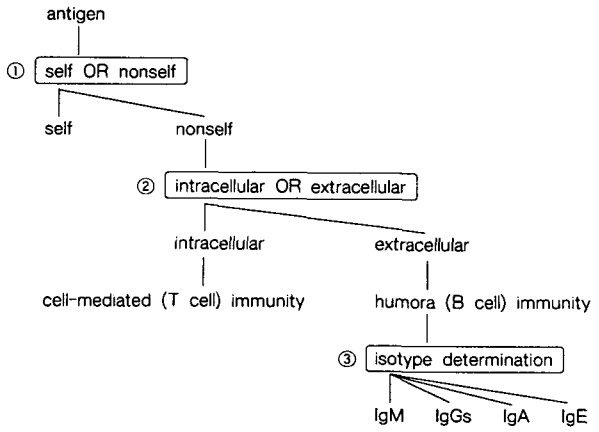

Fig. 1. Three decisions must be made by the immune system for an immune response to be effective: one takes place before exposure to antigen, the other two are made during the course of the immune response.

general class of mononuclear phagocytes. This list is by no means exhaustive. In short, most of the antigen entering the body is eliminated by innate immune mechanisms provided by local phagocytes.

The mononuclear phagocyte is the end of the cellular immune response in the sense that immune effector functions such as immunoglobulin and helper T cells do not directly destroy antigen: the role of the antigen-specific components of the immune system is to capture antigen that has escaped the first innate immune round of phagocytosis and redirect this antigen back to the phagocytic system. Only when biopolymers are reduced to monopolymers are they rendered "safe": the mononuclear phagocyte is the major cell type able to directly destroy antigen.

II. Nitric Oxide Production by Activated Macrophages

In 1818, large amounts of nitrate was reported to be present in the urine of a febrile patient (prout). In 1981, Tannenbaum and colleagues made very much the same observation in human volunteers in nitrite/nitrate balance studies and noted that their excretion of nitrogen oxides exceeded their intake. Green et al used germ-free rats to demonstrate that the host, not its flora, was the source. Working with LPS-treated or BCG-infected mice, Stuehr and Marletta then identified one particular cell as a culprit, the activated macrophage. Hibbs and colleagues implicated the pathway in tumor killing by activated macrophages.

Ironically, the first nonmicrobial species in which production of nitric oxide (NO) was established was the human, and the first cell was the mouse macrophage; yet in humans, the macrophage has been the cell to which the most controversy has attached with respect to its ability to participate in the pathway. Findings in human and mouse now extend to birds, molluscs, horseshoe crabs, insects, protozoa, and slime molds. Macrophage antitumor activity first opened the door to identifying function

and then was nearly brushed aside as evidence rushed in to suggest critical roles in vasoregulation, platelet aggregation, and neurotransmission. The notion that host defense is provided primarily by the specific immune system had to accomodate the finding that most types of nucleated cells can defend themselves with a quintessentially non-specific gas. Initial optimism that a complex biological process-macrophage cytotoxic function-could be accounted for in part by a simple, inorganic compound ran headlong into some of the most unusual and complex biochemistry in enzymology. It is also known that the antimicrobial and cytotoxic actions of NO could be enhanced by other macrophage products such as acid, glutathione, cysteine, hydrogen peroxide, or superoxide.

Although the high-out put NO pathway probably evolved to protect the host from infection, suppre ssive effects on lymphocyte proliferation and damage to other normal host cells confer upon NO from activated macrophages the same protective/destructive duality inherent in every other major component of the immune response.

Ⅲ. Biological Roles of Nitric Oxide

Ⅲ-1. Nitric Oxide, a Novel Biologic Messenger

NO-a small, relatively unstable, potentially toxic, diatomic free radical-has become in the past few years one of the more studied and fascinating entities in biological chemistry. As already cited, this inorganic gas is synthesized by animals as diverse as barnacles, fruit flies, horseshoe crabs, chickens, trout, and humans. It plays a role, often as a biological messenger, in an astonishing range of physiological processes in humans and other animals. Its expanding range of functions already includes neurotransmission, blood clotting, blood pressure control, and a role in the immune system's ability to kill tumor cells and intracellular parasites.

But NO is more than just another-albeit important-biological messenger. It is a new kind of messenger whose trafficking is not dependent on specific transporters or channels used by other chemical messengers. Instead, NO appears to diffuse freely in all directions from its site of origin, making control of its synthesis the key to regulating its activity. The molecule's reactivity, very small size, and diffusibility mean that, more than for any other biological messenger, the actions of NO depend on its chemical properties, rather than its molecular shape.

Before 1981, NO biosynthesis was thought to be restricted to bacteria engaged in nitrification or denitrification reactions. In retrospect, however, there were clues as far back as 1818 that nitrogen oxides were synthesized in mammals, most notably studies showing that there is more nitrate in the urine of rats, pigs, and humans than is present in their diets. These studies were not rigorously confirmed or extended, however, until 1981 when it was demonstrated that nitrogen oxides were quantitatively significant products of mammalian metabolism and that conditions that cause inflammation can also cause a significant increase in nitrate production.

In 1985, NO was first appreciated in mammalian physiology as a mediator of macrophage actions. Macrphages-immune cells that are important in defense against tumors and infections-taken from mice that had previously been injected with an inflammatory mediator or infectious agent produced significant amounts of nitrite and nitrate, whose precusor is arginine. Arginine derivatives such as N^G-monomethyl-L-arginine (N^GMMA) block the formation of NO, as does removal of arginine from the incubation medium. Both these treatments block the tumoricidal and bactericidal actions of macrophages, establishing NO as a crucial mediator of macrophage function (Fig. 2).

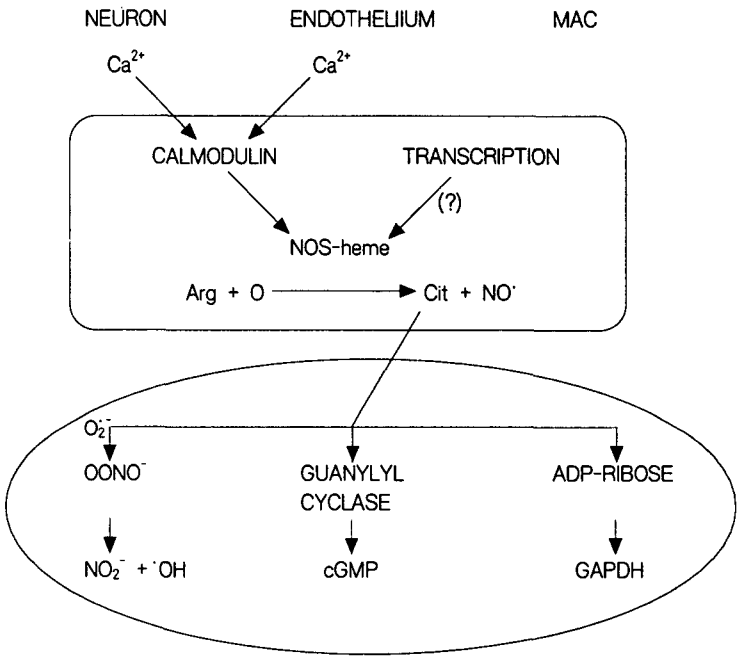

Fig. 2. NO synthesis and mechanisms of action as an intercellular messenger in macrophages, blood vessels, and neurons. MAC, macrophage; cit, citrulline; arg, arginine; NOS, NO synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

A role for NO in blood vessels stemmed from the discovery that the ability of acetylcholine and other agents to relax smooth muscle and hence dilate blood vessels is dependent on the presence of an intact endothelium, which releases a diffusible factor. This endothelial-derived relaxing factor appeared to be extremely labile with a half-life of ~ 5 s. Its identification as NO was facilitated by parallel studies that established NO as the active metabolite mediating the smooth muscle relaxant effects of nitroglycerin and other anti-anginal organic nitrates.

The identification of NO as endothelium-derived relaxing factor led to experiments

showing that vascular tissue can generate NO. Subsequent evidence for NO functions in vasculature derived from knowledge of how NO acts in blood vessels. By binding to iron in the heme at the active site of guanylyl cyclase, NO activates the enzyme to generate cGMP, which stimulated cGMP-dependent protein kinase, resulting in muscle relaxation (Fig. 2). In the brain, the highest densities of cGMP occur in the cerebellum, where the excitatory neurotransmitter glutamate elevates cGMP levels via the N-methyl-D-aspartate (NMDA) subtype of receptors. Glutamate or NMDA increase NO synthase (NOS) activity. Addition of NOS inhibitors such as N^GMMA or N^G-nitro-arginine block both increased NOS activity and elevation of cGMP levels, establishing a role for NO in the neurotransmitter actions of glutamate.

Ⅲ-2. Nitric Oxide Synthase (NOS) in Mammalian Cells

Because NO is so labile, direct measurements of NO are difficult, so that molecular advances have largely stemmed from studies of NOS. There appear to be at least three distinct forms of NOS. Under basal conditions, NOS activity in macrophages is negligible, while stimulation with lipopolysaccharide and interferon-γ produces massive enhancement of NOS in a few hours. Inducible NOS also occurs in neutrophils. Activated neutrophils and macrophages form oxygen free radicals, which can combine with NO to form substances substantially more toxic than NO itself. Thus, NO combined with superoxide anion yields peroxynitrite that decomposes to hydroxide free radical and NO free radical. Since NOS inhibitors completely block macrophage cytotoxic actions, NO generation may be rate limiting, though substances formed by interaction of NO and oxygen free radicals are the cytotoxic effectors (Fig. 3).

In blood vessels and neurons, NOS is constitutive; no major enzyme induction has yet been demonstrated. Instead, the enzyme is activated by calcium, which binds calmodulin as an enzyme cofactor. In blood vessels, acetylcholine stimulates the phosphoinositide cycle generating inositol 1,4,5-triphosphate to release calcium, which binds to calmodulin and activates NOS. In the brain, glutamate stimulation of NMDA receptors opens calcium channels that are part of the receptor protein, triggering calcium influx to elicit increases of NOS.

Ⅲ-3. Structure of NOS and Its Targets

Molecular cloning of NOS has revealed much about its regulation. Neuronal NOS, the first cloned (Bredt et al., 1991), has multiple regulatory sites, including binding sites for NADPH, flavin adenine dinucleotide, and flavin mononucleotide (Fig. 3). Additionally, NOS activity requires tetrahydrobiopterin. Purified NOS contains tightly bound flavin adenine dinucleotide and flavin mononucleotide. The purified enzyme binds heme tightly and absorbs at 450 nm following treatment with carbon monoxide, indicating that NOS is a cytochrome p-450 enzyme. The use of such a large number of cofactors to handle the electron transfers involved in NOS activity is unprecedented.

Another novel feature is the capacity of NOS to produce superoxide as well as NO.

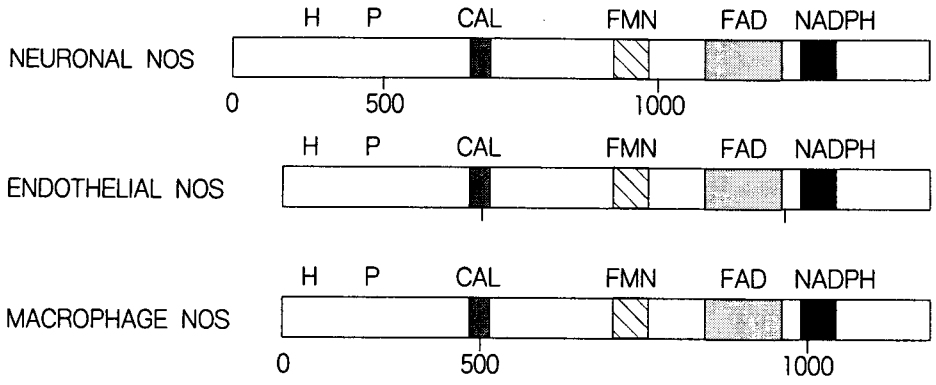

Fig. 3. Structure of neuronal, endothelial, and macrophage NO synthases. CAL, calmodulin binding site; P, consensus site for cAMNP-dependent phosphorylation; H, concensus site for heme binding.

Conceivably, NO and superoxide, both produced by the same enzyme, combine to form the radicals that mediate cytotoxicity.

Neuronal NOS has a recognition site for calmodulin that is also evident in endothelial NOS (Fig. 3). However, in constrast with the dependence of endothelial and brain NOS upon calcium, macrophage NOS activity is calcium independent. Neuronal, macrophage, and endothelial NOS all have consensus sites for cAMP-dependent phosphorylation. Neuronal NOS is phosphorylated by cAMP-dependent protein kinase. The three forms of NOS display about 50% identity in amino acid sequence; macrophage and endothelial NOS are shorter at the N- and C-termini than neuronal NOS.

Ⅲ-4. Nitric Oxide as a Cytotoxic Agent

The role of NO in the immune system is quite different from its function in either neurons or blood vessels. Macrophages contain a third form of NOS that is an inducible enzyme. Many other tissues can express this enzyme as well, including vascular endothelial cells and smooth muscle. In constrast to neuroanl and endothelial NOS, this inducible form of NOS, called iNOS, always contains tightly bound calmodulin, which appears to allow the enzyme to be fully active at basal levels of Ca^{2+}. Thus, once the protein has been synthesized, NO synthesis will occur vigorously as long as substrate is available and the protein remains active.

Transcription of the iNOS gene is controlled, both positively and negatively, by a number of biological response modifiers, called cytokines. The most important positive inducers are interferon-γ, tumor necrosis factor, interleukin-1, interleukin-2, and

lipopolysaccharide, the last of which is not a cytokine but a component of the cell wall of gram-negative bacteria. Interferon-γ often also works synergistically with other cytokines to increase levels of gene transcription. Although, however, over the past few years great attention has been given to a signal transduction pathways that occur during macrophage activation by cytokines and lipopolysaccharide, the exact mechanism of signal pathway regulating the expression and activation of iNOS is not clearly defined (Fig. 4).

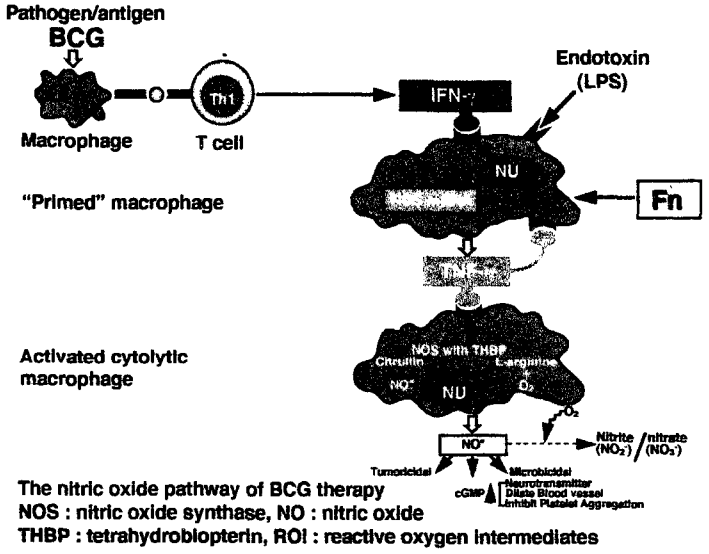

Fig. 4. Schematic model of iNOS induction in murine macrophages by T cell lymphokine, IFN-γ, and bacterial cell wall product such as lipopolysaccharide.

NO produced in stimulated macrophages diffuses into surrounding tissues where it reacts with the iron-sulfur centers of several important macromolecules, including aconitase, an enzyme involved in the tricarboxylic acid cycle, and complex I and complex II of the mitochondrial electron transport chain. NO's high affinity for $Fe^{2}+$ probably results in both the removal of iron from iron-sulfur centers and the formation of dinitrosyl iron species within the proteins involved. Together, inhibition of the activity of these enzymes severely impair the ability of cells to sustain its pool of ATP (Fig. 5).

Several laboratories have shown in animal studies that lipopolysaccharide-induced enzyme may be responsible for much of the hypotension seen in septic shock. The vascular relaxation caused by NO also account, at least in part, for the fact that patients in septic shock often do not respond well to drugs such as dopamine or

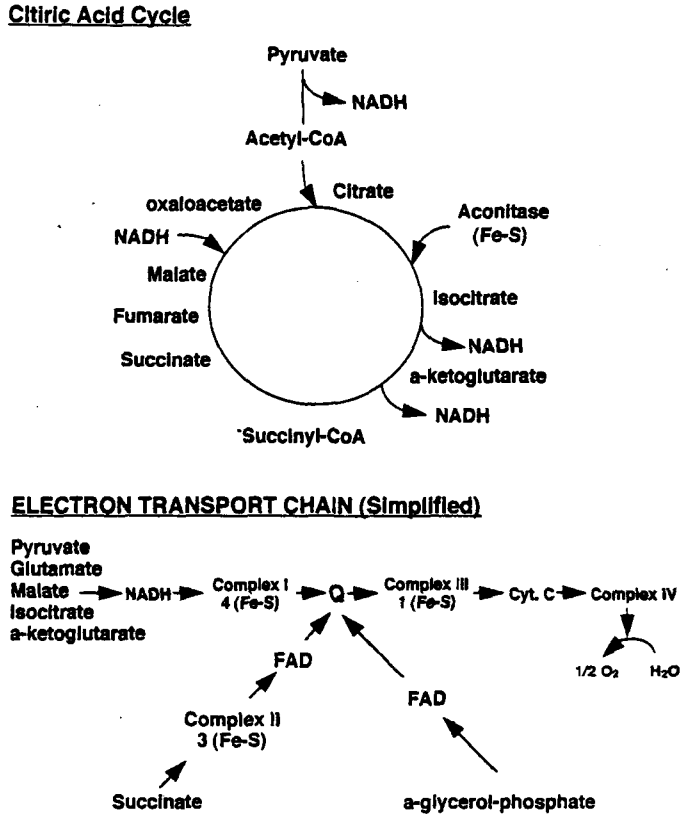

Fig. 5. Schematic representation of the citric acid cycle and the mitochondrial transport chain, NO blocks activities of sulfur-containing enzymes in these diagrams.

norepinephrine that are used to increase blood pressure. The action of NO results in the inability of smooth muscle to respond strongly to any stimulus. Giving NOS inhibitors completely or almost completely restores normal blood pressure in animal models of sepsis. The treatment has been tried in about 30 septic shock patients in Europe and the U.S. Whenever adequate doses of the inhibitors used, blood pressure improved, sometimes to normal, and the patients' response to drugs like dopamine was restored. Although there are concerns about diminished cardiac output and renal perfusion, NOS inhibitors are likely to become an important treatment for hypotension produced by septic shock and by the closely related cytokine-induced shock that occurs in cancer patients being treated with cytokines such as interleukin-1, interleukin-2, or tumor necrosis factor.

Ⅲ-5. The Future Directions

The discovery of the formation of NO from L-arginine by mammalian tissues and the elucidation of some of its biological roles has, in the last 10 years, thrown new light onto many areas of research. NO is released under physiological conditions by a constitutive, Ca^{2+}-dependent enzyme in response to receptor stimulation. This L-arginine: NO pathway is the transduction mechanism for the soluble guanylate cyclase and, as such, is among the mechanisms whereby cells regulate their own function or communicate with others.

The generation of NO also acts as an autocrine regulatory system, for platelets do not seem to transfer NO to other platelets or cells but modulate their own ability to aggregate by generating NO. Furthermore, recent studies show that there may be also self-regulatory mechanism modulating the process of induction and activity of iNOS. NO has been shown to inhibit NOS activity and thus may act as negative feedback modulator of its own synthesis. It is also known that NO and NO generating agent inhibit the activity of protein kinase C (PKC) by S-nitrosylation and the expression of the gene of PKC.

Although NO, originally described in macrophages, has been shown to be cytotoxic or cytostatic for tumor cells and invasive organisms, NO might have other biological consequences, including pathological vasodilation, ADP-ribosylation of target proteins, cell movement, and tissue damage. Immunologically generated NO, in addition to being cytostatic or cytotoxic, may also have similar adverse effects on host cells induced to express the NOS or in the cells adjacent to these. Indeed, macrophages, hepatocytes, and EMT-6 adenocarcinoma cells in which this pathway has been induced show signs of NO-dependent toxicity. The biological consequences of these changes, as well as the circumstances in which the release of NO leads to cell dysfunction or to cell death, await elucidation. However, some forms of local or systemic tissue damage associated with immunological conditions could prove to be related to the release of NO.

In addition to its effects on cell viability and thus proliferation, NO may also play a role in the normal regulation of the response of cells to mitogens. Changes in cyclic GMP have been associated with both the initiation and the control of cell proliferation in many cells. In view of the fact that NO can be released by the constitutive and by the inducible enzyme, it is important to differentiate between actions mediated via the cyclic GMP system and those resulting from NO acting as a cytotixic/cytostatic agent.

Furthermore, previous reports demonstrated that NO, derived as purified gas or released from the pharmacologic NO donors, caused monocytic differentiation of cells such as human leukemic HL-60 cells and altered gene expression. The treated cells stopped proliferating, became spread and vacuolated, and increased expression of nonspecific esterase and the monocytic marker CD14. These reports suggest that NO elaborated in the bone marrow microenvironment might have regulatory roles in normal and malignant hematopoietic programmed cell death or differentiation.

Finally, the implications of the induction of iNOS and NO synthesis in biological system needs to be further analysed and developed. It can, however, be predicted that, as with other fundamantal biological discoveries, this will give us for the understanding of physiological roles of NO in the body.

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