bution to the apparent aggressiveness of breast tumors. Biological studies of a limited number of p53 mutations suggest, for instance, that some p53 mutations are more "oncogenic" than others (6). Similarly, p53 genes having mutations in exons 5 and 6 encode proteins which bind heat shock protein (hsp) 70, whereas exons 7 and 8 mutant proteins do not (14). In this regard, Davidoff et al. (14) have found an association between breast tumors having mutations in exons 5 and 6 and those patients who show a humoral response to p53. In contrast, all of the patients who had a p53-positive tumor, but who were p53 antibody negative, had the mutation in exon 7 or 8. Based on these and other studies, one future direction should be to determine the prognostic significance of p53 mutations as a function of the affected exon or domain.

References


Overproduction of Nitric Oxide in Cytokine-Mediated and Septic Shock

Robert G. Kilbourn,* Owen W. Griffith

Biomedical interest in nitric oxide (NO·) has grown enormously since Furchgott (1) and Ignarro et al. (2) reported at a meeting in 1986 that endothelium-derived relaxing factor (3) is indistinguishable from NO·, a freely diffusible free radical that decomposes spontaneously to nitrite and nitrate. Publications addressing the mammalian biology of NO· now appear at a rate exceeding 2000 per year and have characterized both the origin of NO· and its association with several physiological and pathophysiological processes.

In animal cells, NO· is formed by isoforms of nitric oxide synthase, enzymes catalyzing the O2- and reduced nicotinamide-adenine dinucleotide phosphate-dependent oxidation of l-arginine. The biological effects of NO· appear to be mediated either (a) by activation of guanylyl cyclase, an effect requiring only low concentrations of NO· and normally dependent on constitutive isoforms of nitric oxide synthase or (b) by the potentially cytostatic or cytotoxic destruction of enzymatic iron-sulfur centers, an effect requiring the much higher concentrations of NO· typically produced by inducible isoforms of nitric oxide synthase. Importantly, high concentrations of NO· also activate guanylyl cyclase.

Studies in several species, including humans, indicate that normal blood pressure homeostasis is dependent on basal NO· synthesis by constitutive isoforms of nitric oxide synthase in the vascular endothelium [i.e., classic endothelium-derived relaxing factor formation (4-6)]. In contrast, studies in dogs (7,8) and rats (9) show that overproduction of NO· following cytokine- or endotoxin-mediated expression of inducible nitric oxide synthase can result in inappropriate vasorelaxation and shock.

Although most investigators accept the clinical relevance of animal studies of NO·-mediated shock, reports directly showing induction of nitric oxide synthase in humans or quantitating the resulting NO· synthesis are conspicuously few. The lack of human studies is of genuine concern. While there is little question that the basic enzymology of NO· synthesis will be preserved across species lines, differences among species in their reaction to biological response modifiers are common, and there is substantial evidence that no animal model fully reflects all of the complex physiological changes seen clinically in septic or cytokine-induced shock.

It is in this context that the report by Ochoa et al. (10) in this issue of the Journal, showing increased circulating nitrogen oxides following interleukin-2 (IL-2) immunotherapy, is particularly welcome. These studies and recently reported similar studies by Hibbs et al. (11) are the first to unambiguously show cytokine-mediated induction of NO· synthesis in humans.

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Department of Medical Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas, and Department of Biochemistry, Cornell University Medical College, New York, N.Y.

*Correspondence to: Robert G. Kilbourn, M.D., Ph.D., Department of Medical Oncology, Box 13, Genitourinary Section, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030.
Specifically, Ochoa et al. (10) have shown that plasma nitrate levels are elevated about ninefold in cancer patients receiving IL-2- and anti-CD3-activated lymphocytes. The increase in plasma nitrates correlated temporally and quantitatively (albeit loosely) with the systemic hypotension characteristic of IL-2 administration. Although significant nitrate is present in a normal diet, Ochoa et al. argue convincingly that the increased plasma nitrate seen in these anorexic patients reflects the overproduction of NO- by IL-2-induced nitric oxide synthase. Hibbs et al. (11) achieved even greater verification of this point by directly showing the conversion of [15N]arginine to [15N]nitrate. In an important control study, the possible contribution of decreased renal function to the observed increases in plasma nitrate was shown to be minimal (10). The findings of Ochoa et al. (10) and Hibbs et al. (11) provide strong support for the view that the dose-limiting hypotension associated with the therapeutic use of IL-2 in humans is mediated by NO-.

The studies also accord well with earlier studies by Tannenbaum's group (12), indicating that plasma and urinary nitrate levels increase in endotoxemic animals, an effect now attributable to the endotoxin-mediated induction of nitric oxide synthase (13). It is notable that the findings in IL-2-induced hypotension and in septic shock are mechanistically related, since both IL-2 and endotoxin induce synthesis of IL-1 and tumor necrosis factor (14-16) and those cytokines have been shown to induce nitric oxide synthase in several systems (17,18-19).

Both Ochoa et al. (10) and Hibbs et al. (11) suggest that nitric oxide synthase inhibitors may be useful in controlling the hypotension associated with IL-2 administration. Using Nω-methyl-L-arginine (NMA), a potent and specific nitric oxide synthase inhibitor (20), we have recently completed studies in dogs addressing this point (21). We found that dogs receiving 24 × 10^6 IU of IL-2 daily with concurrent administration of NMA (5 mg/kg per hour) tolerated the therapy well, maintained near-normal blood pressure, and showed no adverse changes in hepatic, renal, or hematological parameters. Survival through the 5-day course was 100%. In contrast, dogs given IL-2 without NMA became premorbid by day 4 and therapy was discontinued. Importantly, studies of lymphocyte-activated killer cell activity in human peripheral blood mononuclear cells (22) and in dogs receiving NMA (23) indicate that the antitumor activity of IL-2 was preserved.

Based in part on these canine studies, a phase I trial of NMA in cancer patients receiving IL-2 therapy is awaiting final approval from the Food and Drug Administration. Preclinical studies of NMA toxicity indicate that the median lethal dose in mice is greater than 2000 mg/kg; doses of 200 mg/kg have no measurable toxicity in dogs (Kilbourn RG, Griffith OW: unpublished data). On the other hand, the maximal antihypotensive effect of NMA appears to be achieved in dogs at an approximate dose of 20 mg/kg. These data suggest a usefully wide therapeutic window. It is anticipated that use of NMA will decrease the toxicity of IL-2 and possibly permit use of increased doses. Since the antitumor effects of IL-2 are dose dependent (24), dose escalation should increase the cure fraction. In demonstrating that NO- production is increased up to ninefold in IL-2 patients, the report by Ochoa et al. (10) demonstrates the relevance of the canine studies to humans and strongly encourages the clinical trial of IL-2 in combination with NMA.

In connection with the prospective clinical use of nitric oxide synthase inhibitors, it noted that enthusiasm for NMA as an antihypotensive agent should be tempered by the recent demonstration that very large doses of NMA increase mortality in endotoxemic rodents (9), an effect not reported in higher species. Other studies indicate that NMA increases hepatic damage following administration of endotoxin to rats (25). While the mechanism of these toxic effects is unknown, it is notable that the relative insensitivity of rodents to cytokines and endotoxin requires their use at very high doses and that the dose-limiting toxicity of cytokines in rodents is different from that observed in higher species and in clinical studies.

The limitations of animal models and the need for human trials are again apparent. Nevertheless, the use of nitric oxide synthase inhibitors in humans should be approached with caution as the necessary pharmacological, toxicological, and metabolic data are gathered during carefully designed phase I clinical trials. The noted pharmacologist Louis Goodman reportedly observed that any new drug discovery will begin with a wave of enthusiasm associated with uncontrolled use, followed by a wave of damnation, ultimately followed by studies that reveal the true state of worth of the drug (Granger D: personal communication). It is hoped that preliminary animal studies (7,8,17,21) and an anecdotal report of the successful use of NMA or Nω-nitro-L-arginine methyl ester (L-NAME) to restore blood pressure in septic shock (26) will not result in the precipitous, uncontrolled, and ultimately counterproductive use of nitric oxide synthase inhibitors in humans.

Although NMA is currently the drug of choice for limiting nitric oxide synthase activity in shock, the biology of NO- synthesis and action is now sufficiently well understood to suggest alternative approaches. Several principles guide the design of both existing and novel therapies. First, the markedly reduced systemic vascular resistance and consequent hypotension seen in septic or cytokine-induced shock appear in all cases to be due to the overproduction of NO- by inducible rather than constitutive isoforms of nitric oxide synthase. Since constitutive isoforms of nitric oxide synthase have a role in neurotransmission (27) and in maintaining normal organ perfusion (28), it is at least theoretically desirable to selectively inhibit inducible nitric oxide synthase or to prevent expression of that isoform. A second key fact is that NO- is freely diffusible and not subject to specific or regulated transport. Although the rapidity with which NO- spontaneously oxidizes to nitrite and nitrate limits its persistence and diffusion, NO- synthesized in one tissue diffuses into and is likely to affect all adjacent tissues.1 This attribute, which is unusual for a biological messenger, assures that the NO- synthesized by any cell or tissue is likely to have multiple physiological and, possibly, pathophysiological effects. It is probable, for example, that NO- produced by activated macrophages has vasorelaxant as well as cytotoxic/cytostatic effects; macrophage-derived NO-

1 The distance over which NO- diffuses in vivo before being oxidized to nitrite or nitrate is a major unresolved question. Although some of the physiological functions of NO- require its diffusion across several cell diameters, the high reactivity of NO- with common biological molecules (e.g., hemoglobin, myoglobin, and superoxide) and the fact that the half-life of NO- is concentration dependent make even rough estimates of its range of action difficult.
may, in fact, be a locally acting, physiologically important hyperemic mediator.

The short half-life but high diffusibility of NO- place some constraint on the tissues likely to be responsible for the overproduction of vasoactive NO- in shock. Although activated macrophages are prototypic inducible nitric oxide synthase-expressing cells, the fact that activated macrophages are limited in number and generally clustered in areas of inflammation suggests, in our view, that they are unlikely to mediate generalized vasodilatation and shock. Similarly, both cytokines and endotoxin have been shown to induce nitric oxide synthase in Kupffer cells and hepatocytes (29), but we do not believe that liver-derived NO- circulates widely enough to cause clinically significant hypotension. In contrast to macrophages or liver, induction of nitric oxide synthase in a widely dispersed cell type would be expected to cause systemic vasodilatation and shock. From this viewpoint, the tissues most likely to account for the overproduction of NO- in shock are the vascular endothelium and vascular smooth muscle. In this regard, Kilbourn and Belloni (18) reported that interferon $\gamma$ in combination with tumor necrosis factor, IL-1, or endotoxin induces expression of nitric oxide synthase in cultured murine brain endothelial cells and suggested that cytokine- or endotoxin-induced expression of nitric oxide synthase in vascular endothelial cells in vivo could account for systemic hypotension (18). Subsequent studies by our collaborators, Gross and Levi (30), and by others (19,31,32) have established that nitric oxide synthase can also be induced in vascular smooth muscle, a tissue which would then both make and respond to NO-.

Considering the total body mass of vascular smooth muscle and the high levels of nitric oxide synthase induction achievable in vitro, this tissue is particularly likely to account for the excess NO- formation seen in septic or cytokine-induced shock.

The considerations outlined above suggest that therapeutic approaches to NO--mediated shock should (a) target the inducible isoform of nitric oxide synthase, (b) accommodate the ready and unregulated diffusion of NO-, and (c) if possible, be particularly effective in limiting NO- production by vascular endothelium and smooth muscle. While approaches meeting all of these goals are not yet evident, the fact that each goal is met by some approaches suggests that combination therapies may be useful. Six distinct approaches to the control of NO- synthesis (I-VI) are considered in more detail.

Approach I: The earliest possible intervention in endotoxic (septic) shock involves the use of antibodies directed against endotoxin itself or against cytokines participating in the cascade initiated by endotoxin (i.e., tumor necrosis factor and IL-1). Although antibodies are of no use in studies such as those of Ochoa et al. (10) where a cytokine is used therapeutically, antibodies show some clinical activity in septic shock (33-35). The advantages of antibody therapy are that all adverse effects of endotoxin can, in principle, be avoided and that constitutive nitric oxide synthase is not inhibited. A major disadvantage is that the antibodies must be given before nitric oxide synthase is induced, since, once induced, nitric oxide synthase may remain active for 24-48 hours (17). In a clinical setting, NO- mediated shock cannot be anticipated with the necessary degree of certainty.

Approach II: Since nitric oxide synthase is a tetrahydrobiopterin-dependent enzyme (36,37), expression of active enzyme requires synthesis of this cofactor. Gross and Levi (38) proposed that inhibition of tetrahydrobiopterin biosynthesis could limit expression of nitric oxide synthase holoenzyme and overproduction of NO-. Specific inhibitors and strategies have been developed (38). Although constitutive nitric oxide synthase is also tetrahydrobiopterin dependent, the presumed slow turnover of that isoform would limit its sensitivity to inhibition of cofactor synthesis; this therapeutic approach is thus effectively specific for the inducible isoform. Tetrahydrobiopterin synthesis inhibitors would, like antibodies, need to be given before nitric oxide synthase induction to be fully effective.

Approach III: Once nitric oxide synthase induction has occurred, therapeutic strategies are best focused on methods limiting enzyme activity. The best characterized inhibitors of nitric oxide synthase are $N^\alpha$-substituted arginine analogues and related compounds for which NMA is the prototype. Because NO- has a short half-life, these agents have the very significant advantage of working rapidly even after enzyme induction has occurred. In both animals and humans, NMA reversed NO-mediated hypotension within a few minutes (7,8,21,26). Since we have shown that the arginine-binding sites of constitutive and inducible nitric oxide synthase have differing affinities for arginine analogues (39), it is likely that selective inhibitors of inducible nitric oxide synthase can be developed. Although inhibitors with true isoform specificity have not yet been reported, it is notable that NMA has some selectivity for the inducible isoenzyme, whereas $N^\alpha$-nitro-L-arginine, the presumed bioactive form of L-NAME, is relatively selective for the constitutive isoform (39). Emphasizing the additional complexities of inhibitor design, $N^\alpha$-amino-L-arginine was found to be a very potent inhibitor but to cause seizures of unknown origin in dogs (40). Furthermore, all of the inhibitors mentioned above appear to block nitric oxide synthase by both reversible and irreversible mechanisms, but it is not yet known which mechanisms are more important in vivo in the tissues of interest (41).

Approach IV: Substrate depletion constitutes another approach to limiting NO- synthesis. Since the limiting substrate in vivo is probably L-arginine, we have explored the use of both intravenous arginine and administration of arginine-free total parenteral solutions as possible therapies. Studies with arginine indicate that basal NO- synthesis, requiring only small amounts of arginine, is unaffected, whereas pathological overproduction of NO- is attenuated (42). If maintenance of intracellular arginine levels in vascular tissues is particularly dependent on plasma arginine, arginine depletion strategies may successfully target the sites of pathological NO- synthesis. Arginine depletion may thus show the desired selectivity with respect to both nitric oxide synthase isozyme and tissue. It should also be noted that overproduction of NO- has been shown to decrease responsivity to the usual pressor agents (e.g., phenylephrine and dopamine) and that inhibition of NO- synthesis using approaches III or IV has been shown to restore sensitivity to these agents (42,43).

Approach V: Another therapeutic approach involves attempts to intercept or prevent the action of NO-. Although a variety of substances are available that either catalyze NO- oxidation (e.g., heme proteins) or sequester NO- (e.g., thiol), therapeutic strategies based on the use of such compounds have been subject to only preliminary explorations. Trapping of extracellular NO- is certainly possible and may have a significant effect on vascular relaxation. However, if vascular smooth
muscle is both the responding tissue and the major source of vasotoxic NO- in shock, interception of NO- before it reacts with guanylyl cyclase will be difficult.

Approach VI: Inhibitors of soluble guanylyl cyclase directly block the vasotoxic effects of NO-. Although methylene blue is reported to inhibit the cyclase and to have a pressor effect in vivo (44), specificity of inhibition has not been established and alternative interpretations are possible.

To summarize, recent reports indicate that overproduction of NO- by an inducible isofom of nitric oxide synthase accounts for septic and cytokine-induced shock in humans (10-13) as had been previously shown in animals (7-9,17,21). Of the several possible therapeutic approaches to controlling NO-mediated hypotension, use of nitric oxide synthase inhibitors (e.g., NMA) has the significant advantage of being applicable to both endotoxin- and cytokine-induced shock, of working rapidly even after nitric oxide synthase induction, and of directly and selectively interfering with the synthesis of the vasotoxic species.

References


(5) Rees DD, Palmer RM, Moncada S: Role of endothelium-derived nitric oxide in the regulation of blood pressure. Proc Natl Acad Sci USA 86:3375-3378, 1989


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