CHAPTER 23

Therapeutic Applications of Nitric Oxide in Infection

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1. Introduction

Nitric oxide (NO) has been an object of intensive investigation as a possible therapeutic agent or target for the treatment of multiple disease conditions ever since it was discovered to be a product of eukaryotic cell metabolism. Indeed, numerous clinical trials have demonstrated that NO, NO donors, or NO scavengers can be used to treat a vast array of circulatory and respiratory ailments. To cite just a few examples, *S*-nitrosoglutathione, nitrate, L-arginine, sodium nitroprusside, and NO gas have been administered orally, topically, parentally, or inhalationally to treat disorders as varied as interstitial cystitis, heart failure, preeclampsia, penile erectile dysfunction, respiratory distress syndrome, and angina pectoris (Berrazueta *et al.*, 1994; Karamanoukian *et al.*, 1994; Langford *et al.*, 1994; Pedrinelli *et al.*, 1995; Wegner and Knispel, 1995). Moreover, several established pharmacological agents (e.g., aspirin, corticosteroids, tetracyclines, cyclosporin) have been only recently discovered to have significant effects on endogenous NO production (DiRosa *et al.*, 1990; Aeberharde *et al.*, 1995; Amin *et al.*, 1995, 1996; Conde *et al.*, 1995; Wu *et al.*, 1995; Walker *et al.*, 1997), suggesting that many longstanding treatment modalities may work, at least in part, via their effects on NO.

Preceding chapters in this volume have documented numerous examples in which NO overproduction can be detrimental during infection, resulting in vascular collapse or tissue injury (Chapters 8,13,19,21). Yet, we have also seen that NO and its derivatives are potent mediators of cellular immunity and constitute an integral

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component of the host's antimicrobial arsenal against many helminths, protozoans, fungi, bacteria, and viruses (reviewed in Chapter 12) (James, 1995), although at the present time there are only a few examples in which the antimicrobial potential of NO has been therapeutically exploited for the treatment of infections. A rapidly growing understanding of the role of NO in infectious processes and the development of an expanding variety of pharmacological NO agonists and antagonists make prospects for NO-based therapy of infection increasingly feasible. In this chapter we present evidence indicating that manipulation of NO can indeed provide therapeutic benefit in infectious diseases.

2. NO Antagonism in the Treatment of Infection

The first application of NO as an antimicrobial agent was probably the addition of nitrites to food products. Because nitrites generate NO, *S*-nitrosothiols, and other reactive nitrogen intermediates, they inhibit microbial multiplication and impart an appealing color to meat via reaction with the heme group of myoglobin. However, despite this well-recognized antimicrobial activity, most NO-related therapeutic interventions in infectious diseases have actually focused on the elimination of pathological side effects arising from NO overproduction.

2.1. Septic Shock

Septic shock (see also Chapters 7 and 13), a syndrome characterized by fever, hypotension, heart failure, tachycardia, tachypnea, respiratory insufficiency, central and peripheral hypoxemia, oliguria, and disseminated intravascular coagulation, typically results from the massive stimulation of monocytes and endothelial cells by microbial cell wall constituents such as LPS, peptidoglycan, or lipoteichoic acid. Excessive quantities of proinflammatory cytokines including IL-1β, IL-6, IL-8, IFNγ, and TNFα are detectable systemically, along with nitrogen oxides. Numerous observations in experimental animal models of septic shock and in infected humans suggest that NO is responsible for many of the hemodynamic alterations that characterize this syndrome (Wright *et al*., 1992; Gomez-Jimenez *et al*., 1995) (see also Chapters 7 and 13). Ever since the initial association of NO with the pathophysiology of septic shock, tremendous attention has been focused on the potential therapeutic benefit of NO synthase (NOS) inhibition in this setting (reviewed in Palmer, 1993; Thiemermann, 1994; Evans and Cohen, 1995; Kilbourne *et al*., 1997a,b).

In support of the hypothesis that NO contributes to the pathology of septic shock, the NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA) increases the blood pressure and systemic vascular resistance in endotoxin-induced septic shock in dogs; similar observations have been made in a limited number of patients with

sepsis (Kilbourn *et al.*, 1990; Petros *et al.,* 1991). However, NOS inhibition normalizes pulmonary arterial pressure in only a minority of septic patients suffering from acute respiratory distress syndrome (Krafft *et al.*, 1996), and the salutary rise in blood pressure is typically accompanied by an undesirable fall in cardiac index (Petros *et al.*, 1994; Mitaka *et al.*, 1995; Jourdain *et al.*, 1997). Although normal blood pressure can be restored in most cases, inhibition of NOS has not increased survival (Wright *et al.*, 1992; Krafft *et al.*, 1996; Park *et al.*, 1996).

One reason for these disappointing preliminary results may be the protective role of low-level NO production by endothelial NOS. Therefore, nonspecific inhibition of all NOS isoforms by inhibitors such L-NMMA or N^G-nitro-L-arginine (L-NNA) may produce both beneficial and detrimental effects. In support of this interpretation, relatively specific inducible NOS (iNOS) inhibitors such as L-canavanine or *S*-methylisothiourea both stabilize blood pressure and increase survival in endotoxemic rats or mice, effects that are not observed when nonselective inhibitors are used (Szabo *et al.*, 1994; Teale and Atkinson, 1994; Liaudet *et al.*, 1998) . Also, mice genetically deficient in iNOS have reduced mortality and hypotension induced by LPS (Nathan, 1995; Wei *et al.*, 1995). Thus, selective inhibition of the iNOS isoform may be a more appropriate therapeutic approach to septic shock. The recent structural resolution of the iNOS oxygenase domain and inhibitor complexes should expedite the development of novel selective inhibitors (Crane *et al.*, 1997). As discussed in Chapter 7, the dosage and timing of drug administration and the patient's fluid status appear to be additional important factors determining the benefit of NOS inhibition during sepsis. Another possible confounding factor is the role of NO as an antimicrobial mediator (Chapter 12); beneficial hemodynamic effects of NOS inhibition could be counterbalanced by enhanced microbial proliferation. However, the combination of effective antimicrobial therapy and iNOS inhibition may circumvent this problem. Indeed, Teale and Atkinson (1992) found that NOS inhibition is beneficial in an experimental model of bacterial peritonitis when effective antibiotics are coadministered.

An alternative to inhibiting NO synthesis is the removal of NO from the circulation. Maeda *et al.* (1995) have shown that imidazolineoxyl *N*-oxide, an effective NO scavenger, can prolong the survival of LPS-treated rats. Increased survival coincided with an improvement in the mean arterial pressure. Iron chelates have been used in a murine model to bind NO and decrease sepsis-associated mortality (Kazmierski *et al.*, 1996). Hemoglobin is another NO scavenger that has been used to treat the sepsis syndrome. Hemoglobin can undergo *S*-nitrosylation as well as heme–NO interactions (Gow and Stamler, 1998). In rat or ovine models of endotoxic shock, polymerized hemoglobin has been shown to restore mean arterial pressure and heart rate without interfering with renal function, in contrast to the NOS inhibitor L-NNA (De Angelo, 1997; Heneka *et al.*, 1997). Polymerized

hemoglobin remains in the circulation as a consequence of its high molecular weight, which may permit NO in interstitial and intracellular compartments to continue mediating physiologic NO actions. Preliminary observations in healthy volunteers, patients with septic shock, or patients receiving adjunctive cytokine therapy for cancer encourage further evaluation of polymerized hemoglobin for the treatment of sepsis-related hypotension (De Angelo, 1997; Kilbourn, 1997; Reah *et al*., 1997), although the enhanced mortality following coadministration of LPS and hemoglobin to mice emphasizes the need for caution (Su *et al*., 1997).

Another alternative therapeutic approach in settings of NO overproduction is the downregulation of the NOS enzyme itself. For example, the protection against LPS-induced shock conferred by tetracycline or doxycycline appears to result from a decrease in IL-1β, TNFα and iNOS expression (Milano *et al*., 1997). Abnormally high TNFα and IL-1β production are hallmarks of septic shock; therefore, considerable effort has been spent in developing anticytokine-based therapies (Dinarello, 1995) that can indirectly reduce NO production. However, treatment with certain cytokines may also be beneficial in septic shock. In a murine model of posttraumatic sepsis, increased survival conferred by GM-CSF correlated with decreased macrophage NO-producing capacity (Austin *et al*., 1995).

Because of the diverse etiologies of septic shock, the complex interactions of the multiple inflammatory mediators produced, and the complex roles of NO in this syndrome, it is unlikely that NO inhibition alone will provide a panacea for sepsis. Nevertheless, selective iNOS inhibitors may well become an important component of a multifaceted therapeutic approach in the future.

2.2. Other Infections

Although not as extensively investigated as septic shock, NO also contributes to the immunopathology of many other infectious diseases (e.g., Chapters 8, 19–21) (Khan *et al*., 1997). The pathology of whooping cough can be mimicked *in vitro* by tracheal cytotoxin, a muramyl peptide produced by *Bordetella pertussis.* Goldman and collaborators have reported that tracheal cytotoxin triggers epithelial NO production, leading to autodestruction of the epithelium (Heiss *et al*., 1994; Flak and Goldman, 1996). The NOS inhibitors L-NMMA and aminoguanidine can attenuate the ciliostasis and epithelial cell death caused by tracheal cytotoxin (Heiss *et al*., 1994), raising the possibility that NO inhibition might ameliorate the clinical manifestations of whooping cough *in vivo.*

The therapeutic potential of NOS inhibition has also been investigated in animal models of acute viral pneumonitis. Excessive production of NO elicited during influenza virus infection appears to play a crucial role in the associated respiratory tract pathology (Akaike *et al*., 1996; see Chapter 18). Akaike and colleagues have provided evidence that nitrotyrosine, an oxidative signature of peroxynitrite or certain other NO congeners, accumulates in macrophages,

neutrophils, and intraalveolar exudate from influenza-infected lungs. Treatment with L-NMMA improved the survival of the mice with influenza pneumonitis, without affecting viral replication. In a murine cytomegalovirus-associated immune-mediated pneumonitis model, NO antagonism was found to be beneficial despite the absence of tyrosine nitration, suggesting that NO overproduction can be detrimental for lung tissue even in the absence of peroxynitrite formation (Tanaka *et al.*, 1997). Inhibition of NO synthesis has been shown to decrease lethality in a murine model of herpes simplex virus (HSV) pneumonitis (Adler *et al.*, 1997), despite *in vitro* evidence that NO is a potent inhibitor of HSV replication (Croen, 1993; Karupiah *et al.*, 1993; Komatsu *et al.*, 1996). In contrast to the absence of an effect on viral replication seen in the murine influenza model, NOS inhibition *in vivo* coincided with a significant augmentation of the HSV viral burden (Adler *et al.*, 1997). Nevertheless, L-NMMA treatment resulted in increased survival, increased pulmonary compliance, and decreased lymphocyte infiltration. Upregulation of iNOS is also observed in HSV encephalitis (Meyding-Lamade *et al.*, 1998), a devastating condition in which antiviral therapy has limited efficacy and immunomodulatory intervention is highly attractive. Khan and co-workers have recently shown that NOS inhibition reduces early mortality and tissue injury associated with acute toxoplasmosis in mice, despite an associated enhancement of parasite replication (Khan *et al.*, 1997). Together, these results illustrate that the importance of NO's immunopathological effects can supersede its antimicrobial actions in certain infections.

Deleterious effects of NO overproduction in chronic infections should not be overlooked. Infectious agents including *Helicobacter pylori, Schistosoma haematobium,* hepatitis C virus, and *Opisthorchis viverrini* have been strongly correlated with both NO overproduction and carcinogenesis (Ohshima and Bartsch, 1994; Warren *et al.*, 1995; Satarug *et al.*, 1996; Tsuji *et al.*, 1996; Kane *et al.*, 1997). Eradication of *H. pylori* from gastric lesions using a combination of antimicrobial agents and antioxidants can reduce iNOS expression and nitrotyrosine formation in the gastric mucosa (Mannick *et al.*, 1996). Because the overproduction of NO has been proposed to be a genotoxic mechanism leading to the development of cancer, reduction of NO synthesis related to chronic infection could have far-reaching clinical implications (Bartsch *et al.*, 1992; De Koster *et al.*, 1994; Fox, 1994).

3. Nutritional Modulation of NO-Mediated Host Resistance

The disproportionally high incidence of tuberculosis in developing countries and in immunosuppressed individuals may be partly attributable to malnutrition. An iNOS-dominated immune response correlates with a favorable prognosis in mice and humans suffering from tuberculosis (Nicholson *et al.*, 1996; MacMicking *et al.*, 1997b). In an interesting report, Chan *et al.* (1996) recently demonstrated that

malnourished mice infected with *M. tuberculosis* exhibit a reduced granulomatous reaction, low expression of iNOS in pulmonary tissue, and an increased mycobacterial burden. These signs were reversed after nutritional supplementation, suggesting that proper nutrition can boost NO-mediated antimicrobial immunity. Such noninvasive measures might dramatically reduce the incidence of tuberculosis and other "opportunistic" pathogens in impoverished populations throughout the world.

The risk of microbial translocation from the gastrointestinal tract to systemic sites may also be amenable to nutritional NO-related intervention. Dietary supplementation with L-arginine can improve the survival of mice suffering from sepsis-related experimentally induced peritonitis or extensive burns (Gianatti *et al.*, 1993; Gennari and Alexander, 1997; Horton *et al.,* 1998). Although the enhanced resistance observed in L-arginine-supplemented mice could be related to a nonspecific stimulation of a T-cell-mediated immunity (Barbul *et al.*, 1980; Kirk *et al.*, 1992), reversal of L-arginine's salutary effects by the NOS inhibitor L-NNA strongly suggests that NO is involved (Gianatti *et al.*, 1993).

4. Indirect NO Antimicrobial Therapy

Many cytokines as well as some transduction pathways regulating expression of iNOS have been identified (MacMicking *et al.*, 1997a) (Chapter 5). Although a comprehensive review of the modulation of cytokines for the treatment of disease is beyond the scope of this chapter, it must be considered that certain effects of these therapies are likely to be mediated by NO. Some investigators have shown that genetic or immunological depletion of cytokines can abrogate iNOS expression and increase susceptibility to infection (Kimura *et al.*, 1994). Similarly, cytokine therapy can enhance resistance to infection. To cite just a few examples, GM-CSF, IFNγ, or IL-12 therapy can stimulate a robust NO response and increase host resistance to *Candida albicans*, *Histoplasma capsulatum*, *Cryptococcus neoformans*, *Leishmania donovani*, or *L. major* in animal models (Hill *et al.*, 1995; Lovchik *et al.*, 1995; Kawakami *et al.*, 1997; Taylor and Murray, 1997; Zhou *et al.*, 1997). IFNα has been demonstrated to induce iNOS expression in human mononuclear cells during treatment of patients with hepatitis C infection (Sharara *et al.*, 1997) (Chapter 6), and the beneficial effects of IFNα in this infectious condition could be attributable to its effects on endogenous NO production. Xu *et al.* (1998) have recently used the novel approach of expressing migration inhibitor factor, IL-2, IFNγ, and TNFα from recombinant attenuated *Salmonella* strains; these constructs were able to enhance endogenous iNOS expression and reduce parasite burden in *Leishmania*-infected mice.

The action of amphotericin B, the antifungal agent of choice for many opportunistic systemic infections including candidiasis, cryptococcosis, blastomycosis, and histoplasmosis (Abu-Salah, 1996), has traditionally been associated with its capacity to interfere with the synthesis of ergosterol, a cholesterol-like constituent of the fungal membrane. More recently, it has become evident that amphotericin B also possesses immunomodulatory properties. Amphotericin B-dependent stimulation of macrophage TNFα and IL-1 production (Yamagushi *et al.*, 1993; Louie *et al.*, 1994; Tohyama *et al.*, 1996) along with enhancement of iNOS expression appears to be required for its anticryptococcal activity in an *in vitro* macrophage model (Tohyama *et al.*, 1996). Ironically, these immunomodulatory actions may partially account both for amphotericin B's antifungal actions and for its undesirable systemic side effects.

5. Direct NO Antimicrobial Therapy

Although *in vitro* and *in vivo* studies have demonstrated that reactive nitrogen intermediates possess broad-spectrum antimicrobial activity (Chapter 12), few researchers have yet investigated the direct therapeutic potential of these compounds. NO-related antimicrobial activity can potentiate the effects of other antimicrobial agents. For example, in an *in vitro* system, diazenium diolate NO-donors were shown to synergize with fluconazole, miconazole, or ketoconazole against strains of *C. albicans*, *C. krusei*, *C. parapsilosis*, and *C. tropicalis* (McElhaney-Feser *et al.*, 1997).

By inhibiting the enzyme ribonucleotide reductase, hydroxyurea blocks deoxynucleotide synthesis and interferes with HIV-1 replication (Lori *et al.*, 1994). This observation has prompted the evaluation of hydroxyurea in combination with other antiretroviral agents for the treatment of HIV-infected patients, with encouraging preliminary results (Rossero *et al.*, 1997). Hydroxyurea has been shown to eradicate Epstein–Barr virus episomes in *in vitro* experimental models (Chodosh *et al.*, 1998), suggesting that this agent might also be useful in other viral infections. Noting the structural similarity with N^{ω}-hydroxy-L-arginine, Kwon *et al.* (1991) have investigated whether hydroxyurea might be generating NO, a known potent inhibitor of ribonucleotide reductase. In fact, catalyzed either by hydrogen peroxide and a transition metal or by hemoproteins, NO can be formed from hydroxyurea (Kwon *et al.*, 1991; Pacelli *et al.*, 1996). These studies suggest that at least some of the antiviral activity of hydroxyurea might be mediated by NO, and should prompt the investigation of additional NO-based antiviral therapeutic strategies.

An encouraging multinational clinical study conducted by Drs. Patricio Lopez-Jaramillo, Salvador Moncada, and collaborators has tested the therapeutic potential of the NO donor *S*-nitroso-*N*-acetyl-penicillamine (SNAP) used

topically in cutaneous infectious diseases. Application of a cream containing 200 μM SNAP to cutaneous lesions caused by the fungi *Trichophyton tonsurans*, *T. mentagraphytes*, *Epidermophyton floccosum*, or C. *alhicans*, or by the protozoan *Leishmania mexicana*, resulted in both an improvement in clinical signs and the resolution of infection as demonstrated by sterilization of the affected site (Lopez-Jaramillo *et al.*, 1995, 1998). A double-blind and more extensive study assessing the therapeutic applications of topical SNAP is now under way in Ecuador. Acidified nitrite cream has also been used for the treatment of tinea pedis (Weller *et al.*, 1998). The accessibility of skin lesions makes cutaneous infection a particularly attractive setting in which to test the feasibility of NO-based antimicrobial therapy. Treatment of infection at other tissue sites may need to await the development of more sophisticated drug delivery strategies.

6. Conclusions

Our understanding of the complex roles of NO in infection has advanced remarkably during the past decade. However, the practical application of this knowledge to the prevention or treatment of infection has only scratched the surface. A better understanding of the NO-producing host cells and tissues that participate in the immune response to infection, the NO congeners that mediate immunopathology or host resistance to infectious diseases, the microbial species and critical molecular targets of specific NO metabolites, and the mechanisms that microbes use to avoid or resist NO congeners will ultimately contribute to the rational utilization of NO-based antimicrobial therapies. Emerging problems with resistant or refractory infections (Neu, 1992; Ash, 1996; Gold and Moellering, 1996; Nicolle *et al.*, 1996) make novel NO-based approaches attractive as therapeutic alternatives. Utilization of NO-based therapies will be further facilitated by the development of new NO donors, NO scavengers, and selective NOS inhibitors.

Delivery systems to target specific organs or tissues may also expedite the use of NO-modulating drugs for the therapy of localized infectious diseases, and might lessen many of the unwanted side effects associated with NO therapies. One step toward this objective has been achieved by Saavedra and collaborators, who successfully engineered a drug, l-(pyrrolidin-l-yl)diazen-l-ium-l,2-diolate, to deliver NO specifically to the liver (Saavedra *et al.*, 1997).

Initial applications of NO-based therapies have focused on cardiovascular and respiratory conditions. It is exciting to contemplate the expansion of this therapeutic revolution to the realm of infectious disease in the near future.

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