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## Review Article

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# Inducible Nitric Oxide Synthase and Inflammatory Diseases

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### Introduction

Nitric oxide (NO) is a colorless gas at room temperature and one of the simplest molecules known, yet it has been implicated in a wide variety of regulatory mechanisms ranging from vasodilatation and blood pressure control to neurotransmission. It is also involved in non-specific immunity and participates in the complex mechanism of tissue injury as a major mediator of inflammatory processes and apoptosis (1). This work focuses on the complex role of NO produced by the inducible form of nitric oxide synthase (iNOS) in inflammatory and autoimmune diseases. Although the earliest studies in the field suggested that NO is a strictly pro-inflammatory macrophage product, it is clear from the current literature that, in fact, NO is made by numerous cell types and is often anti-inflammatory. Much of this dichotomy can be explained by the particular responses of given cells involved in the inflammatory response, but another variable involves the complex chemistry in which NO can participate. As we outline below, various facets of the immune response can be examined from these perspectives.

### Nitric Oxide and Inflammation

The physiological defense response of the body to any kind of injurious stimulus is called inflammation. There is no clear dividing line between acute and chronic inflammation, but

the former generally refers to a response that has an abrupt onset and is of short duration. Acute inflammation may become chronic (in the temporal sense) if the injurious agent is persistent. On the other hand, chronic inflammation is characterized by a proliferation of fibroblasts and formation of blood vessels (angiogenesis), as well as an influx of chronic inflammatory cells, namely granulocytes (neutrophils, eosinophils, and basophils), lymphocytes, plasma cells and macrophages. [See (2) for a comprehensive work providing an up-to-date look at the basics of inflammatory processes.]

Nearly two decades ago, the production of nitrogen oxides was associated with inflammation. It was already known in 1981 that a marked increase in urinary nitrate excretion occurs in humans with diarrhea and fever (3). Nitrate formation was then believed to be a result of microbial metabolism, but these observations suggested that mammals also form nitrogen oxides and that a correlation between immunostimulation and nitrate synthesis may exist (4). In 1985, production of nitrite and nitrate-generating compounds by mammalian cells in vitro was first demonstrated in the mouse macrophage (5). Since that time, the production of NO has been considered of primary importance in the host's antimicrobial mechanisms.

The metabolic pathway known as the L-arginine:NO pathway is the main source for the production of NO in mammalian cells by a group of enzymes known as the nitric oxide synthases (NOS). Endothelial cells and neurons express isoforms of NOS (eNOS and nNOS, respectively), which produce NO at low levels under the physiological control of the Ca<sup>2+</sup>/calmodulin system, will not be discussed

extensively in this review. The enzyme primarily responsible for the roles of NO in inflammatory processes is the inducible NOS (iNOS; NOS2; or type II NOS), which is not typically expressed in resting cells and must first be induced by certain cytokines or microbial products. The molecular biology and regulation of NO synthases have been reviewed extensively (6,7). We would like to highlight that iNOS remains very stable at both the mRNA and protein levels, and generates large amounts of NO over a period of days (6,8,9). At least two general conclusions can be gleaned from these studies and numerous others. First, sustained production of NO at high levels will lead to the production of numerous reactive nitrogen oxide species (RNOS), which can mediate a broad spectrum of physiological and pathological effects (10). Second, due to the possibility of deleterious effects to the host as a consequence of prolonged exposure to such RNOS, iNOS must be regulated carefully (11). Finally, one can hypothesize that microorganisms may have developed means for suppressing the expression and/or activity of iNOS, perhaps by co-opting the host's own regulatory machinery. Viewed from this perspective, the balance between induction and suppression of iNOS may underlie much of the physiology and pathology of inflammation.

In recent years, NO has emerged as a major mediator of inflammation. As might be expected from such a pleiotropic molecule, there are contradictory reports in the literature concerning its role as an anti-inflammatory or pro-inflammatory agent. The inconsistencies reported probably are due to the multiple cellular actions of this molecule, the level and site of NO production, and the redox milieu into which it is released. Therefore, the type, concentration, and flux of RNOS. Nitric oxide itself activates soluble guanylyl cycles, which leads to synthesis of cGMP. This activation of soluble guanylyl cyclase constitutes a common pathway in many processes, including vascular smooth muscle cell relaxation, inhibition of platelet activity, inhibition of neutrophil chemotaxis, and signal transduction in the central and peripheral nervous systems (12). As stated above, under both physiological and pathological conditions the reaction of NO with ROS, for example superoxide, results in the formation of RNOS. These agents are directly involved in the activation or inhibition of key enzymes in various metabolic processes, such as

mitochondrial respiration and DNA synthesis and repair, as well as in the modulation of various genes (for reviews see 9,10, 13–17). Many of the regulatory and physiological functions of NO (Table 1) can be considered as protective or “anti-inflammatory,” and are mainly related to NO produced by the other isoforms of NOS. However, in the last years, data have accumulated about iNOS expression in an increasing number of human disorders.

### **Interactions of NO with the Chemical Mediators of Inflammation**

Inflammation is controlled by the presence of a group of chemical mediators, each with a specific role at some definite stage of the inflammatory reaction. These mediators may be exogenous, arising from bacteria or chemical irritants, or endogenous in origin. The most important endogenous mediators identified include the vasoactive amines histamine and serotonin, the kinin system, the fibrinolytic system, the complement system, the arachidonic acid metabolites like prostaglandins and leukotrienes, platelet-activating factor (PAF), neuropeptides, reactive oxygen species, and inflammatory cytokines (2). We will discuss only those components of inflammation directly related to the actions of NO.

### **Inducible NOS and Inflammatory Cytokines**

Cytokines are small-molecular weight proteins comprising regulatory factors of the immune system, hematopoiesis, tissue repair, cell proliferation and inflammation. It has been reported that in different cell types, in vitro, so-called pro- and anti-inflammatory cytokines can have both enhancing and suppressing effects on the expression of iNOS and NO production. The biological activities of cytokines *vis-à-vis* iNOS expression have been mainly investigated in vitro and in vivo by using purified or recombinant proteins and neutralizing antibodies, but the use of genetically modified animals gives better insights as to the roles of cytokines in experimental diseases. For example, disruption of the transforming growth factor-beta 1 (TGF- $\beta$ 1) gene in mice resulted in a severe wasting syndrome with multifocal inflammation and early death (18). In these TGF- $\beta$ 1 null mice, systemic NO production was greatly elevated over that of

**Table 1. Regulatory and anti-inflammatory actions of NO**

Tissue Organ	Physiological Action of NO Related to Inflammation	NOS Isoform	Refs.*
Vascular endothelium	— Maintains vasodilator tone	eNOS	(239)
	— Inhibits smooth muscle cell migration and proliferation	eNOS; iNOS	(240,241)
	— Inhibition of blood cell-vessel wall interactions and adhesion to endothelium	eNOS	(242)
Blood cells	— Inhibition of platelet adhesion and aggregation, and inhibition of microvascular thrombosis	eNOS; iNOS	(243,244)
		eNOS	(242)
	— Prevents aggregation and adhesion of white cells	iNOS	(245)
	— Mediates cytostatic and cytotoxic activity of macrophages for antimicrobial and antitumor defense	iNOS	(9,246)
Heart	— Inhibits mast cell degranulation		(247,248)
	— Maintains coronary perfusion and regulates cardiac contractility	eNOS	(249)
	— Inhibits cardiac contractility (pathology of myocarditis)	iNOS	(250,251)
Lung	— Maintains ventilation/perfusion ratio and regulates bronchociliar motility and mucus secretion	?	(252–254)
Pancreas	— Modulates endocrine secretion		(255,256)
Intestinal system	— Modulates peristalsis and exocrine secretion	eNOS, iNOS	(257)
	— Contributes to protection of mucosa	eNOS	(258,259)
			(208,260)
			(261,262)

\* The quoted references are mostly recent reviews of significant relevance. eNOS, endothelial isoform of nitric oxide synthase (NOS); iNOS, inducible form of NOS; NO, nitric oxide.

wild-type littermates, in association with aberrant iNOS expression in multiple organs (19).

The activated macrophage is one of the most important effector cells in the inflammatory response. In addition to NO (9), macrophages secrete pro-inflammatory cytokines including tumor necrosis factor (TNF- $\alpha$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ ) and immunomodulatory cytokines, such as IL-2, IL-10, TGF- $\beta$ 1, and IL-6 (20,21). IL-4 (22), IL-6 (23), IL-10 (24) and TGF- $\beta$ 1 (8) have been reported to suppress the induction of NO from macrophages or to down-regulate the expression of iNOS in activated macrophages, but the list continues to grow. In a murine model of endotoxemia, human recombinant IL-11 attenuated the inflammatory response through down-regulation of pro-inflammatory cytokine release and NO production (21). Furthermore, IL-13 was recently found to suppress macrophage NO production in both mouse peritoneal macrophages and J774 macrophage cell line. Regulation of iNOS occurred at both the mRNA and translational levels, depending

on the macrophage population (25). In the case of IL-13, its similarity to IL-4 in its spectrum of actions may suggest that IL-4 and IL-13 overlap with regard to suppression of iNOS, as well.

Since the release of cytokines constitutes a major event in inflammatory and immune responses, their opposing effects on the production of NO may partially explain why pro-inflammatory cytokines induce their detrimental effects, while anti-inflammatory cytokines may have beneficial effects in inflammation. A recent study showed that the levels of pro-inflammatory (TNF- $\alpha$ , IL-6, IL-8) and anti-inflammatory cytokines (IL-10, TNFsrI, TNFsrII) relate to serum nitrate levels in patients with severe sepsis. An excessive production of pro-inflammatory cytokines was related to an excessive production of NO in the acute phase of sepsis; whereas, during the secondary phase, the production of NO was reduced and the anti-inflammatory cytokines predominantly were present (26). Although administration of exogenous anti-inflammatory

cytokines, such as IL-10, to septic patients may possibly lead to diminished NO production, the efficacy of this treatment remains to be established due to the possible protective role of NO in sepsis. Our group demonstrated a decade ago that inhibition of systemic NO production with the nonselective NOS inhibitor N<sup>G</sup>-monomethyl-L-arginine (L-NMMA) in endotoxemic mice was associated with increased liver damage (27). In a similar vein, Cobb et al. (28) found increased mortality following treatment of conscious endotoxemic dogs with another nonselective NOS inhibitor, N-omega-L-arginine. This paradox was illustrated further in a recent study of endotoxemia in TGF- $\beta$ 1 transgenic mice, in which mortality was higher in the transgenic animals, compared with controls, in conjunction with a greatly suppressed systemic NO production. Paradoxically, TGF- $\beta$ 1 transgenic animals also expressed very high circulating levels of TNF- $\alpha$ , which might explain this increased mortality (29). Indeed, sepsis and septic shock are complex, though their symptoms can be mimicked to a degree by administration of lipopolysaccharide (LPS) and/or TNF- $\alpha$ .

TGF- $\beta$ 1 negatively regulates iNOS expression both in vitro and in vivo (19), but endogenous and exogenous TGF- $\beta$ 1 can act differently to suppress NO production (30). Though overexpression of endogenous TGF- $\beta$ 1 was associated with the aforementioned increase in endotoxin-induced mortality, exogenous TGF- $\beta$ 1 has been reported to reduce the expression of iNOS, improve hemodynamic parameters, and decrease mortality of endotoxemic rats (31,32). Thus, the fact that a single cytokine may display opposite effects in different experimental models has to be considered when evaluating a possible therapeutic use of recombinant cytokines.

Nitric oxide may have an important regulatory role in the process of cytokine activation. Nitric oxide was recently reported to be a potent inhibitor of cysteine proteases, such as IL-1 $\beta$ -converting enzyme (33). NO suppressed IL-1 $\beta$  and interferon- $\gamma$  (IFN- $\gamma$ )-inducing factor (IGIF or IL-18) processing in activated RAW 264.7 mouse macrophages by inhibiting caspase-1 activity (34). Furthermore, stimulated peritoneal macrophages from wild-type mice released more IL-1 $\beta$  if exposed to the NOS inhibitor L-NMMA; whereas, macrophages from iNOS null mice did not (34). This indicates that regulation of pro-inflammatory cytokines

release by iNOS may contribute to the pathogenesis of certain inflammatory processes. On the other hand, NO could lead to the indirect activation of TGF- $\beta$ 1, possibly through suppression of the capacity of latency-associated peptide to neutralize TGF- $\beta$ 1 (35). In this way, a negative feedback cycle may be established by which iNOS expression could be reduced in the presence of high levels of NO.

### Inducible NOS and Arachidonic Acid Metabolites

It is known that prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) is a regulator of macrophage functions and displays a functional dualism in immunoinflammatory conditions (36). The expression of inducible NO synthase after stimulation by bacterial endotoxin and other cytokines is accompanied by the release of other mediators, such as PGE<sub>2</sub> and prostacyclin, via the cyclooxygenase (COX) pathway (37,38). This synergistic production has been the subject of several studies (39,40), which suggests a crucial link between the NO synthase and cyclooxygenase pathways in certain pathological conditions, such as nephrosis, sepsis or rheumatoid arthritis (38). Most studies have focused on the role of NO in the expression and/or activity of cyclo-oxygenase (38,41). NO has been reported to increase prostaglandin production via activation of both constitutive and inducible forms of COX in a number of cell types (38,42–44). Moreover, a recent study showed the existence of both NO-dependent and -independent pathways of prostaglandin synthesis after cytokine stimulation of rat osteoblasts in vitro (45). On the other hand, NOS inhibitors increased PGE<sub>2</sub> synthesis in Kupffer cells (46) and chondrocytes (47). More recently, two unrelated NO donors, namely GEA 3175 and S-nitroso-N-acetyld,L-penicillamine (SNAP), were shown to inhibit prostacyclin production in human umbilical endothelial cells (48).

The effect of eicosanoids on the NO synthesis by the activation of the inducible NO synthase also has been studied (49–51). After stimulation of macrophages with bacterial endotoxin plus IFN- $\gamma$ , induction of iNOS and NO production is accompanied by the release of prostaglandins via the cyclo-oxygenase pathway (37,38). Like many other laboratories, we showed that incubation with LPS plus IFN- $\gamma$  led to a dose-dependent production of NO in murine J774 macrophage-like cells, an effect

prevented by the NOS inhibitor L-NMMA. Addition of the cyclo-oxygenase inhibitor, indomethacin, did not affect  $\text{NO}_2^-$  production significantly (44). These findings indicate that the products of the cyclo-oxygenase pathway do not play a major role in the regulation of iNOS and confirm previous studies, which demonstrate that the endogenous release of prostanoids from the RAW 264.7 and J774.2 murine macrophages is insufficient to affect the activity of iNOS (38,41). However, the effects of prostaglandins on iNOS activity are still controversial. Low concentrations of indomethacin have been reported to reduce NO formation significantly (51) and the amount of iNOS protein (52) in LPS-stimulated J774 macrophages. Also, in LPS plus IFN- $\gamma$ -stimulated J774 macrophages, a significant reduction in NO production could only be found when indomethacin was used at very high concentrations (44). Similarly, anti-inflammatory drugs, such as aspirin and sodium salicylate, have been shown to inhibit induced NO production by immunostimulated RAW 264.7 cells at the high end of therapeutic concentrations. Moreover, this effect was not simply the result of inhibition of prostaglandin synthesis, because exogenous  $\text{PGE}_2$  failed to overcome the effects of both drugs (53,54). In another study, high doses of aspirin inhibited IL-1 $\beta$ -induced iNOS protein expression in bovine vascular smooth muscle cells and decreased NF- $\kappa$ B translocation and TNF- $\alpha$  production. This study suggests new mechanisms of action for aspirin in the treatment of cytokine-induced inflammatory diseases (55).

In a recent study, inhibition of endogenous  $\text{PGE}_2$  synthesis with indomethacin or ibuprofen had no effect on NO synthesis (56). Thus, the inhibitory effects of the high concentration of COX inhibitors like indomethacin have to be interpreted with caution. Interestingly, exogenous, but not endogenous,  $\text{PGE}_2$  decreased the levels of iNOS mRNA and iNOS protein in LPS-stimulated RAW 264.7 cells. This inhibition of macrophage iNOS expression was shown to be dependent on the time and concentration of prostaglandin exposure (56).

## Nitric Oxide in Acute Inflammation

### *Inducible NOS and the Vascular Response to Injury*

Injury to an organ or tissue results in progressive changes in the damaged area. As the result

of vascular alterations in the area, three main signs of vascular response appear: redness, heat, and swelling. The redness and heat result from an increase in blood flow, which is the result of local vasodilatation, first involving arterioles and then capillaries and venules. The production of NO by the eNOS in endothelial cells activates soluble guanylyl cyclase, leading to the synthesis of cyclic guanosine monophosphate (cGMP), which in turn leads to relaxation of vascular smooth muscle cells. This pathway has been investigated extensively and constitutes a common process in both human and many animal tissues (57). Swelling is the result of alterations in vascular permeability. The endothelial cells become leaky, leading to exudation of fluid, plasma proteins and white blood cells (inflammatory edema).

The carrageenan-induced edema model has been a useful experimental tool with which to assess the contribution of mediators involved with the vascular changes associated with acute inflammation and for screening efficacious anti-inflammatory drugs (58). The development of carrageenan-induced edema in the rat hindpaw is a biphasic event in which the early phase is related to the production of histamine, leukotrienes, PAF, bradykinin, and possibly cyclo-oxygenase products. The delayed phase is linked to local neutrophil infiltration and activation. The contribution to edema of NO, superoxide and peroxynitrite also has been demonstrated in this model (59). Both the nonselective NOS inhibitors  $N^G$ -nitro-L-arginine methyl ester (L-NAME) and L-NMMA (at the early phase), and the selective iNOS inhibitors  $N^G$ -iminoethyl-L-lysine (L-NIL) and mercaptoethylguanidine (MEG) (at the late phase) have a potent inhibitory effect, which strongly suggests a pro-inflammatory effect for both constitutive and induced NO production (58,60). However, the location and identity of the NOS isoforms responsible for NO synthesis at the site of inflammation remains to be determined. In this context, a recent study using the selective nNOS inhibitor 7-Nitroindazole (7-NI) suggests that NO synthesized by a nNOS isoform located in sensory nerves plays an important part in the early phase response to carrageenan in this model of inflammation. In addition, NO synthesized by an iNOS isoform located in inflammatory leukocytes contributes to the late phase response (61). Interestingly, injection of carrageenan into the pleural cavity of mice reduces the induction of iNOS protein

in both macrophages and airway epithelial cells in the lungs of both IL-6 null mice, as well as in wild-type mice pretreated with an antibody against IL-6. This finding suggests that endogenous IL-6 amplifies the induction of iNOS caused by carrageenan in the lung (62).

Neutrophils are the first leukocytes to emerge from the vessels in significant numbers during acute inflammation. Although the carrageenan-induced paw edema is neutrophil-dependent and mediated by both the NOS and COX pathways (60), no evidence is found for the involvement of either cyclo-oxygenase products or neutrophils in mediating the iNOS inflammatory component in a model of dermal inflammation (63). In a recent study, the development of an inflammatory reaction induced by injection of specific agonists of proteinase-activated receptor-2 (PAR2)-activating peptides in the rat hindpaw was shown to be largely independent of the production of prostanoids and NO (64). However, as part of the zymosan-induced inflammatory response in the rat skin, NO contributed to edema formation by increasing blood flow. The sources of iNOS appeared to be cells other than neutrophils. It was suggested that other cell types, such as dermal fibroblasts and keratinocytes, that are also known to express iNOS, could be important sources of NO in the skin (65).

This process of leukocyte recruitment initiates with the adhesion of leukocytes to the endothelium, an event regulated by a series of adhesive interactions. Activated endothelium expresses surface adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), and E-selectin, which interact with peripheral blood leukocytes and facilitate their attachment to the endothelial cell surface. The inhibitory effects of endogenous NO in endothelial-leukocyte interaction were shown previously (66). Recently, a novel physiologic mechanism was identified by which macrophage-derived NO can inhibit endothelial VCAM-1 expression and modulates the activation of resident and nonresident vascular wall cells in an autocrine and paracrine manner (67,68). While administration of LPS to wild-type mice increased sequestration of neutrophils in the lung and their adhesion to the endothelium, these responses were markedly exaggerated in mice lacking iNOS (69). Although this suggests a beneficial effect for iNOS expression, in other experiments, the ex-

pression of iNOS had no impact on the mobilization of leukocytes into the peritoneal cavity induced by a number of inflammatory irritants such as thioglycollate broth or LPS plus IFN- $\gamma$  (70). Similarly, in an oyster glycogen-induced model of acute peritonitis in rats, the iNOS-specific inhibitor, L-NIL, significantly inhibited NO production without altering polymorphonuclear neutrophils (PMN) recruitment, compared with vehicle-treated rats. The authors concluded that PMN-associated, iNOS-derived NO does not play an important role in modulating extravasion of these leukocytes *in vivo* in this model of acute inflammation (71).

The putative formation of peroxynitrite from NO during nitrosative stress may cause DNA single-strand breakage, which stimulates the activation of the nuclear enzyme poly(ADP-ribose) synthetase (PARS). Rapid activation of PARS depletes the intracellular concentration of its substrate, nicotinamide adenine dinucleotide (NAD<sup>+</sup>), slowing the rate of glycolysis, electron transport and subsequent formation of ATP. This process can result in acute cell dysfunction and cell death (72,73). PARS also regulates the expression of a number of genes, including the genes for ICAM-1, collagenase, and iNOS. Inhibition of PARS protects against zymosan- or endotoxin-induced multiple organ failure, arthritis, allergic encephalomyelitis and diabetic islet cell destruction (72). Inhibition of PARS reduced neutrophil recruitment and reduced the extent of edema in zymosan- and carrageenan-triggered models of local inflammation (74). Furthermore, PARS null mice were more resistant against inflammation and organ injury than wild-type animals. Part of the anti-inflammatory effects of PARS inhibition were attributed to a reduced neutrophil recruitment, which may be related to maintained endothelial integrity (75).

### Inducible NOS in Acute Inflammatory Responses

Sepsis and septic shock are caused by bacterial infection and represent an acute systemic inflammatory response. Septic shock is characterized by systemic hypotension, vascular smooth muscle hyporeactivity to adrenergic mimics, and myocardial depression (76,77). Cellular activation by cell wall components of Gram-negative or Gram-positive bacteria results in the production of a variety of inflammatory mediators that are essential for the development of

septic shock and its complications (26). Nitric oxide is crucial in the pathogenesis of septic shock (70,78–82).

Infusion of endotoxin or TNF- $\alpha$  into experimental animals is used to mimic septic shock and systemic NO production (83). Several studies showing an antihypotensive and protective effect for NOS inhibitors in rodent models of septic shock (31,84,85) suggest that the inhibition of NO production could be useful for the treatment of this condition. In contrast, in a murine model of chronic hepatic inflammation (administration of *Corynebacterium parvum* followed by LPS), our group has consistently shown the deleterious effect of NOS inhibition causing liver damage, intravascular thrombosis and oxygen radical-mediated hepatic injury (27, 86–88). The use of NOS inhibitors also has led to controversial results. In a murine model of endotoxemia, the nonselective NOS inhibitor, L-NAME, enhanced liver damage and tended to accelerate the time of death; whereas, the iNOS selective inhibitor, L-canavanine, significantly reduced mortality and had no deleterious effects in terms of organ damage (89). L-NAME also aggravated liver damage in a rat model of endotoxemia, while the iNOS selective inhibitor S-methylisothiourea did not increase LPS-induced damage (90). Inhibition of iNOS with L-NMMA also resulted in reduction of Cu/Zn SOD expression levels in rat glomerular mesangial cells treated with LPS. This finding suggests that up-regulation of Cu/Zn SOD by endogenous NO may serve as a protective mechanism against formation of peroxynitrite or other potentially damaging RNOS, such as nitroxyl anion (91), in conditions associated with iNOS induction during endotoxic shock (92).

Recently, mice lacking the iNOS gene were reported to be protected against LPS-induced mortality (70,93). However, another study demonstrated that mice lacking iNOS were not resistant to LPS-induced death (94). In two models of endotoxic shock in iNOS and respiratory burst oxidase deficient mice, major reductions in the ability to form NO or superoxide also failed to improve survival (95). Recently, Cobb et al. (96) used iNOS null mice to examine the effect of inducible NO production in a clinically relevant model of polymicrobial abdominal sepsis treated with antibiotics. The survival study showed that iNOS gene deficiency increased the mortality of sep-

sis in mice, suggesting a beneficial role for iNOS gene function in septic mice.

These contradictory effects of septic insults in iNOS null mice may be explained by modulation of NO production in the liver. The liver is a major site of the response to endotoxin (97). The dual effects of NO, both as hepatoprotective and hepatotoxic agent (98), and its role as a bifunctional regulator of apoptosis (99) were reviewed recently.

Nonspecific inhibition of iNOS also has been reported to be detrimental, rather than beneficial (27,83,100,101). Recent results showing that iNOS-deficient mice have enhanced leukocyte-endothelium interactions in endotoxemia raised the possibility that induction of iNOS is a homeostatic regulator for leukocyte recruitment (69). Although iNOS expression can protect the liver in acute hepatic inflammation, it may account for hepatic necrosis in ischemia/reperfusion and hemorrhagic shock (98). In a murine model of hemorrhagic shock, it was found that the expression of iNOS and NO production caused an increase in PMN influx, activation of the transcriptional factor NF- $\kappa$ B, and upregulation of IL-6 and granulocyte colony stimulating factor (G-CSF) mRNA levels. These changes were associated with marked lung and liver injury [(102), see also a recent review on the novel roles of NO in the pathogenesis of hemorrhagic shock and resuscitation (103)]. Thus, factors, such as the cellular redox status, the production of reactive oxygen species and pro-inflammatory cytokines, the type of insult, and the isoform selectivity of different NOS inhibitors, will determine the possible therapeutic use of NOS inhibitors in the future.

A critical role for the transcription factor NF- $\kappa$ B has been demonstrated in the transcriptional regulation of the murine and human iNOS gene induced by LPS and cytokines in cultured cells (104–107). More recently, it was reported that LPS activated NF- $\kappa$ B in vivo, which, in turn, induced transcription of the iNOS gene and expression of the iNOS protein in a rat model of septic shock. The authors suggested that targeting NF- $\kappa$ B might be a more effective strategy for the treatment of septic shock, because inhibition of NF- $\kappa$ B activation selectively prevented the increase in iNOS activity and iNOS-mediated NO production (108). Of course, this hypothesis is based on the putative detrimental role for NO in sepsis. In a recent study, treating poly(ADP-ribose)

polymerase-1 (PARP-1)-deficient mice with LPS did not result in the rapid activation of NF- $\kappa$ B seen in macrophages from wild-type mice. The PARP-1 null mice were extremely resistant to LPS-induced endotoxic shock, which was explained by the almost complete abrogation of NF- $\kappa$ B-dependent accumulation of TNF- $\alpha$  in the serum, as well as the down-regulation of iNOS (109). This study suggests PARP-1 may be a possible target for therapeutic interventions.

## Nitric Oxide in Immunity and Chronic Inflammation

### *Inducible NOS and the Specific Immune Response*

Release of NO has been reported in inflammatory responses initiated by microbial products or autoimmune reactions. Although the role of NO in nonspecific immunity is well-established in animal models (9), it still awaits definitive confirmation in humans. The effects of NO on specific immunity, however, need extensive investigation. In the generation of an inflammatory response, the defensive machinery of the immune system is based mainly on the activity of effector cells, such as T lymphocytes, macrophages and neutrophils. The activation of these cells results in the production of immune modulators, including cytokines, chemokines, and reactive oxygen and nitrogen species that form a complex regulatory network that determine the intensity and duration of inflammation. Based on the cytokine secretion pattern of CD4<sup>+</sup> helper T lymphocytes, two main subsets of T helper cells are defined (110): T helper type I (Th1) and T helper type II (Th2). The former are mainly implicated in cell-mediated immune reactions, macrophage activation and the production of opsonizing antibodies. At least in mice, this subset of T cells secrete IL-2, IFN- $\gamma$ , and TNF- $\beta$ . Th2 cells, on the other hand, secrete IL-4, IL-5, IL-6, IL-10 and IL-13, and are key players in humoral immunity and activate mast cells and eosinophils (110,111).

Several relevant diseases that are positive for iNOS also exhibit pro-inflammatory, Th1-type cytokine expression or cytokine response profiles (112,113). As is the case with sepsis, the elevated expression of iNOS in affected tissues suggests that iNOS is involved in the pathogenesis of certain immune diseases, but a number of controversial reports in the literature again suggest a possible dual role for NO. Although NO generation from L-arginine was

required for DNA synthesis in human peripheral blood lymphocytes in one study (114), another study showed that human lymphocytes did not produce the appreciable amounts of NO needed to affect lymphocyte mitogenesis (115). Furthermore, the inhibitory effects of two NO donors (sodium nitroprusside and nitroglycerin) on lymphocyte function were shown to be nonspecific and unrelated to NO production (115). Recently, allogeneic (mixed leukocyte cultures), mitogenic and superantigenic stimulation of bovine blood mononuclear cells induced NO production at a low level and without having any effect on cellular activation and proliferation (116).

In fact, most of the existing data suggest that NO suppresses, rather than enhances, lymphocyte activation and proliferation. Antigen-stimulated mouse Th1 cells produce high levels of NO that result in a concomitant reduction of IL-2 secretion and lymphocyte proliferation. This is reversed by addition of recombinant IL-2 (117). Moreover, there is evidence that NO exerts different effects on discrete subpopulations of T cells, for example by inhibiting secretion of IL-2 by murine Th1 cells and increasing secretion of IL-4 by Th2 cells (118). This preferential effect, however, was not observed in activated human T cells and human T-cell clones *in vitro*, where the Th1- and Th2-associated cytokine production was equally impaired by the NO donors SIN-1 and SNAP (119). The question of whether NO differentially affects T lymphocyte function merits special attention, because the outcome of numerous diseases appears to depend critically on the Th1/Th2 balance in accompanying immune responses (111,113). Nitric oxide may amplify inflammation by altering this balance. Furthermore, the increased proliferation of Th2 lymphocytes may, in turn, produce a cytokine profile that was associated with exacerbation of asthma. These observations, however, have not been extended yet to humans, where the Th1/Th2 paradigm is less well-defined (120).

Both cGMP-independent and -dependent pathways have been described to explain the antiproliferative effects of NO. In human lymphocytes activated by lectin mitogen concanavalin (ConA), two oxotriazole derivatives (GEA 3162 and 3175) and the nitrosothiol SNAP caused inhibition of cell proliferation and enhanced cGMP production. While a guanylyl cyclase inhibitor inhibited the NO donor-induced cGMP production, the antipro-



liferative action remained unaltered (121). In contrast, another study showed that T cells activated in the presence of alveolar macrophages were unable to proliferate, despite the expression of IL-2 receptor and secretion of IL-2. The NO-mediated T cell suppression was reversible by the guanylyl cyclase inhibitors, methylene blue and LY-83583, and was reproduced by a cell-permeable analogue of cGMP. In addition, this effect could be reproduced by the addition of SNAP and inhibited by the NOS inhibitor L-NAME (122).

The involvement of NO production by iNOS in important autoimmune diseases, such as immunologically induced diabetes, inflammatory arthritis and graft versus host disease (GVHD), appears to be unquestionable. However, because of differences in the experimental animals and disease-induction methods, it is unclear whether NO is beneficial or detrimental. In several studies, administration of selective iNOS inhibitors to rodents with autoimmune diseases, led to conflicting results. One possible explanation for these often contradictory results is that iNOS inhibition is detrimental to the host during priming of pathogenic T-cell responses in the periphery, but largely protective at the site of disease (123). Early studies in a rodent model of arthritis suggested that selective inhibition of iNOS was beneficial in this disease state (124). In a different study of rats with adjuvant arthritis, however, administration of the NOS inhibitor, L-NIL, was without effect (125). Moreover, the protective role for NO in the host response to infections with *Staphylococcus aureus* strongly cautioned against the clinical use of selective NOS inhibitor therapy in diseases such as septic arthritis (126).

Experimental allergic encephalomyelitis (EAE) is a well-studied animal model of organ-specific autoimmunity that mimics human multiple sclerosis. Treatment with aminoguanidine ameliorated EAE in both mice and rats (127–129), but it led to aggravation and prolongation of disease in myelin and T cell-mediated EAE (130). Similarly, L-NIL administration caused a marked worsening in disease expression in myelin basic protein (MBP)-immunized Lewis rats, but ameliorated the severity of disease following adoptive transfer of MBP-reactive T cells into L-NIL-treated recipients. Also in Lewis rats, aminoguanidine was shown to ameliorate ex-

perimental autoimmune myocarditis (131). Detrimental effects for iNOS inhibition also were reported in a model of autoimmune interstitial nephritis (132) and experimental autoimmune uveoretinitis (EAU) (133). More recently, it was reported that IL-12 protected mice from Th1-mediated EAU through a mechanism involving IFN- $\gamma$ -induced NO production and bcl-2-regulated apoptotic deletion of the antigen-specific T cells (134).

To address the limitations of the administration of NOS inhibitors, iNOS null mice and antisense nucleotides were used to identify the functional roles of iNOS in different pathologies. In a model of EAE, mice deficient in or with reduced iNOS activity showed more disease and less remission than wild-type mice (135). In contrast, intraventricular administration of antisense oligodeoxynucleotide complementary to iNOS to SJL/S mice significantly reduced the clinical score of EAE and blocked the iNOS mRNA, the protein synthesis and the iNOS activity within the CNS (136). More recently, inhibition of allergic airway inflammation was observed in mice lacking iNOS (137).

The consequences of iNOS disruption were studied in MRL-lpr/lpr mice. These mice produce an excess of NO and develop a systemic autoimmune disease associated with a number of inflammatory manifestations, like glomerulonephritis, arthritis and vasculitis. iNOS-disrupted and wild-type mice displayed equivalent degrees of nephritis and arthritis, but the former showed markedly reduced vasculitis, suggesting heterogeneity in mechanisms of inflammation in MRL-lpr/lpr mice (138). Similar results showing that, in iNOS null mice, glomerulonephritis did not differ from that in mice with an intact iNOS gene, suggest that iNOS does not play an essential role in this autoimmune disease in the mouse (139).

Nitric oxide may also affect inflammation that occurs due to organ transplantation. Acute rejection is an immunoinflammatory process characterized by an intense inflammatory cell infiltrate and progressive destruction of the grafted organ. Although it has become apparent that NO contributes to allograft rejection, GVHD and tissue damage in alloimmune responses (140,141), the complex regulatory and effector mechanisms that underlie the rejection process are not completely understood. Production of NO was shown to partially account for the destruction of both lymphoid and erythroid host tissue, as well as the reduced lym-

phoproliferative responses associated with the acute phase of GVHD in mice (142). Also in mice with acute GVHD, induction of the NOS pathway by TNF- $\alpha$  was reported to suppress B-cell proliferation (143). A recent study showed that NO production by non-T, non-B, L-leucine methyl ester-sensitive cells mediated the graft versus host reaction-associated, IFN- $\gamma$ -dependent immunosuppression of T-cell proliferation and of antibody synthesis by CD5(+) B cells (144). Administration of NOS inhibitors in models of GVHD was reported to prolong graft survival in mice undergoing GVHD (145), or to have no effect at all in mice receiving allogeneic heterotopic heart transplants (146). Administration of the nonselective NOS inhibitor, L-NMMA, produced only a small increase in graft survival in a model of heterotopic cardiac transplantation in the rat (147). Generation of NO also was observed in acute rejection of rat hepatic allografts (148) and of pancreas allografts in hyperglycemic rats, where electron spin resonance measurement of NO was suggested as a useful marker for the diagnosis of acute rejection in pancreas transplantation (149).

In humans, expression of iNOS was localized in lung transplant recipients with obliterative bronchiolitis (150) and in the coronary arteries of transplanted human hearts with accelerated graft arteriosclerosis. The role of NO in the pathogenesis, however, remains unknown (151). In transplanted rat aortic allografts, the inhibition of NO production significantly increased the intimal thickening, suggesting that NO suppresses the development of allograft arteriosclerosis (152). In the same study, transduction with iNOS using an adenoviral vector completely suppressed the development of allograft arteriosclerosis, indicating that iNOS may be important for the suppression of transplant vasculopathy in chronic rejection associated with cardiac transplantation (152). It was recently reported that cardiac myocyte apoptosis was closely associated with expression of iNOS in macrophages and myocytes and with nitration of myocyte proteins by peroxynitrite during human cardiac allograft rejection (153). Moreover, it was suggested that NO plays a role in modulating the localized bone resorption that accompanies the aseptic loosening of prosthetic joints (154). The identification of iNOS in the setting of organ transplantation will certainly open novel possibilities for the use of selective inhibitors and pharmacological intervention in

the treatment of these alloimmune conditions. Other relevant immunologic effects attributed to NO are the inhibition of lymphokine-activated killer-cell induction by inducing apoptosis of cytolytic lymphocyte precursors (155); inhibition of major histocompatibility class II expression on mouse peritoneal macrophages and antigen presentation by lung dendritic cells; tumor-induced immunosuppression; and the reduced immunological response resulting from administration of morphine (13).

The demonstration of the involvement of NO in a number of autoimmune and inflammatory diseases does not necessarily imply that NO itself is the effector molecule. Whether these pathologies are directly mediated by NO or by RNOS, such as peroxynitrite or nitroxyl anion, requires further investigation. Inflammatory mediators that enhance the cellular production of NO also increase cellular superoxide production from various cellular sources, including NOS itself (156). Nitration of tyrosine, thought to occur due to the production of peroxynitrite and assessed by the degree of formation of nitrotyrosine, is used as a footprint of peroxynitrite activity (157–160). The putative role of peroxynitrite as an inflammatory mediator has been suggested after detection of nitrotyrosine in animal models of endotoxemia (161–163), lung injury (164,165), ileitis (166,167), experimental autoimmune encephalomyelitis (168, 169), myocardial ischemia-reperfusion injury (170,171), myocardial dysfunction (172), and glomerulonephritis (173), as well as in human atherosclerotic plaques (174,175), adult respiratory distress syndrome (176), airways of asthmatic patients (177), multiple sclerosis (178, 179), and human sepsis and myocarditis (180). However, it is still not clear whether the apparent requirement for the simultaneous presence of equimolar concentrations of NO and superoxide to form peroxynitrite (181) and, subsequently, form nitrotyrosine residues on proteins (182) can be fulfilled under pathologically relevant circumstances. Furthermore, recent evidence suggests that both myeloperoxidase (183,184) and eosinophil peroxidase (185) can oxidize NO<sub>2</sub><sup>-</sup> to form nitrotyrosine, which suggests pathways of nitrotyrosine formation independent of peroxynitrite may exist.

#### *Inducible NOS in Chronic Inflammatory Diseases*

Chronic inflammation is characterized by a proliferation of fibroblasts and small blood vessels, as well as an influx of chronic in-

flammatory cells (lymphocytes, plasma cells, macrophages). In certain immunologic conditions, chronic inflammation is primary and not preceded by an acute inflammatory response. It also differs from acute inflammation in that it is orchestrated almost entirely by cells of the immune system (2). Although more tightly regulated than the rodent iNOS gene, expression of human iNOS has been found in chronic inflammatory diseases of the airways, the vessels, the bowels, the kidney, the heart, the skin and the apex of teeth (112), strongly indicating that NO plays an important role in the pathogenesis of chronic inflammation. Table 2 summarizes the most relevant chronic inflammatory conditions related to NO in humans. Below, we discuss some of the relevant studies both in humans and in animal models.

The involvement of NO in chronic localized inflammatory diseases has been demonstrated in a number of experimental animal models. Nitric oxide stimulates TNF- $\alpha$  production by synoviocytes and its catabolic effects on chondrocyte function promote the degradation of articular cartilage implicated in certain rheumatic diseases (16,186). Most studies indicate that NO is at least partly responsible for IL-1-induced suppression of glycosaminoglycan and collagen synthesis (187). In human chondrocytes, IL-18 has been identified as a cytokine that regulates chondrocyte responses and contributes to cartilage destruction through stimulation of the expression of several genes, including iNOS, inducible COX, IL-6, and stromelysin (188). The beneficial effects of inhibition of NOS and scavenging of NO have been shown in murine systemic lupus erythematosus (SLE) (16,189), suppression of rat adjuvant arthritis by  $N^G$ -iminoethyl-L-ornithine (L-NIO) (190) or L-NMMA (191), attenuation of streptococcal cell wall-induced arthritis in rats by L-NMMA (124), reduction of inflammation and injury in synovial tissue from joints with inflammatory arthritis by hemoglobin (192), prevention of chondrocyte death and cartilage erosion by local IL-4 application in the knee joint of mice with collagen-induced arthritis (193), and the suppression by diphenylene diodonium chloride of potassium peroxocromate-induced arthritis in mice (194). Cyclic tensile stress, which mimics the tensile stress experienced by chondrocytes on the surface of cartilage during movement, was also found to suppress pathologic effects of IL-1 $\beta$  through inhibition of inducible NO production

**Table 2. Nitric oxide in human autoimmune and chronic inflammatory diseases**

Disease	Reference
Rheumatic diseases	
Systemic lupus erythematosus	(263–265)
Vasculitis	(266,267)
Rheumatoid arthritis	(264,268–271)
Osteoarthritis	(186,268, 272–274)
Inflammatory airway disease	
Asthma	(177,230, 275–277)
Respiratory tract infections	(278–282)
Idiopathic pulmonary fibrosis	(283–285)
Bronchiectasis	(286–288)
Gastrointestinal system	
Inflammatory bowel disease	(289)
Ulcerative colitis	(290–293)
Crohn's disease	(290–292,294)
Diverticulitis	(290,295)
Necrotizing enterocolitis	(296–298)
Celiac disease	(299–302)
<i>Helicobacter pylori</i> -associated chronic gastritis	(303–306)
Kidney	
Glomerulonephritis	(307–310)
Lupus nephritis	(310–312)
Pancreas	
Diabetes	(313–316)
Pancreatitis	(317)
Liver	
Chronic hepatitis	(318,319)
Bladder	
Infectious and noninfectious cystitis	(320–323)
Central and peripheral nervous system	
Parkinson's disease	(324–326)
Multiple sclerosis	(327–330)
Severe AIDS dementia	(331–333)
Vasculitic and optic neuropathy	(334,335)
Skin	(336)
Psoriasis	(337–340)
Cutaneous lupus erythematosus	(341)
Systemic sclerosis	(342–344)
Dermatitis	(345–347)
Atherosclerosis	(219–221, 348–350)
Periapical periodontitis	(351–353)
Sjögren's syndrome	(354–356)

in primary rabbit chondrocytes in vitro (195). Furthermore, in a model of osteoarthritis (OA) in dogs, inhibition of NOS reduced the progression of cartilage lesions and the production of metalloproteinases and IL-1 (16). In syn-

oviocytes from OA patients, two NO donors (SNAP and sodium nitroprusside) markedly increased p53 protein expression and DNA fragmentation in vitro (196). This suggests that iNOS-derived NO may be a major inducer of synoviocyte apoptosis in OA in vivo. Recently, iNOS-deficient mice were used to investigate the role of NO and IL-1 in joint inflammation and cartilage destruction in a nonimmunologic model of inflammation, the zymosan-induced gonarthrosis (197). In this study, IL-1 and NO played only a minor role in edema and neutrophil influx, but a major role in cartilage destruction. Moreover, the results obtained from anti-IL-1 treatment of wild-type mice were comparable to those found in iNOS null mice, which suggests that most IL-1-related effects in arthritis were mediated by NO (197).

Although most experimental findings suggest that the actions of NO in the cartilage are detrimental, there is also evidence for protective functions of NO. In a recent study, intravenous inoculation with *S. aureus* induced significantly increased clinical severity of septic arthritis, with attendant septicemia in iNOS-deficient mice, compared with similarly infected heterozygous or wild-type mice. This was associated with enhanced production of IFN- $\gamma$  and TNF- $\alpha$  in vivo and in vitro, which indicated a shift towards increased production of Th1-type cytokines (126). Apart from antimicrobial activity, other beneficial effects of NO include stimulation of proteoglycan synthesis during certain conditions, participation in wound healing, and stimulation of collagen production (187).

The NO-mediated destruction of both rat and mouse islets of Langerhans and its effects on insulin secretion provide strong evidence for the involvement of NO in human diabetes (112). Administration of a natural IL-12 antagonist, which suppressed the progression of islet inflammation and concomitant upregulation of iNOS (198), and overexpression of the anti-apoptotic gene A20, which abrogated cytokine-induced NO production and protected both human and rat islet cells against apoptosis (199), suggest possible strategies for therapeutic intervention against NO-mediated toxicity in islet inflammation. However, it is not known whether the inhibition of human iNOS will reduce the destruction of >90% of the pancreatic islets found in type-1 diabetes.

NO also contributes to mucosal damage in inflammatory bowel disease (200,201) and

the beneficial effects of NOS inhibitors for reducing intestinal inflammation is shown in various models of colitis (202–204). Furthermore, NO also is reported to promote mucosal integrity (205). The isoform nonselective NOS inhibitor L-NAME worsens acute edematous and necrotizing pancreatitis; whereas, NO donors reduces pancreatic injury (206). Indeed, there is increasing evidence that iNOS is beneficial, rather than detrimental, for resolving intestinal inflammation (207). Evidence for the dual roles of inducible NO in modulating gastrointestinal mucosal defense and injury is presented in a recent review (208).

The relationship between inflammation and atherosclerosis is well established, but the biologic events that trigger the local inflammatory response within plaque are not fully understood. The development of atherosclerosis and hyperlipaemia per se is accompanied by impairment of endothelium-dependent vasodilation. Atherosclerosis is associated with marked changes in the activity of NOS isoforms in the artery wall, including increased expression of the iNOS in complex human lesions, as well as in the neointima of experimental animal models. Defective NO production by eNOS, together with inducible NO and superoxide anions generated by inflammatory cells, are detrimental events that may cause apoptosis and injury to both the endothelium and myocytes, and possibly lead to plaque rupture. In this way, the balance between the possible protective effects of NO and the deleterious effects of RNOS may be disturbed (10). In endothelial cells, NOS prevents apoptosis (209,210); whereas, it induces apoptosis in smooth muscle cells (211–215).

The presence of iNOS in atherosclerotic plaques suggests a role for NO in atherosclerosis (216–218), but its exact role is still unknown. Inducible NOS and nitrotyrosine is detected within the atheroma (219), and COX-2 and iNOS/nitrotyrosine co-localize predominantly in macrophages/foam cells in both native and transplanted human coronary arteries (220). These findings may suggest an ongoing production of both prostanoids and RNOS, such as peroxynitrite, both of which may have proatherosclerotic effects. Interestingly, high expression levels of the anti-inflammatory cytokine, IL-10, are associated with significant decreases, in iNOS expression and cell death in a recent study of advanced human atheroscle-

rotic plaques (221). Additionally, TGF- $\beta$ 1 and its signaling system are perturbed in atherosclerosis (222–226). These findings suggest that a balance between iNOS-inducing and iNOS-suppressing mediators might modulate the expression of iNOS in atherosclerosis.

In situ hybridization and immunohistochemical techniques localize iNOS to human lung in certain disease states. The expression of iNOS is described in a murine model of allergic asthma (227), foreign body-induced granulomatous lung inflammation (228), as well as in radiation pneumonitis and fibrosis in rats (229). Exhaled NO levels are increased in patients with asthmatic flares, bronchiectasis, and active tuberculosis, and are considered as a marker of inflammatory injury; however, the precise role of NO in lung inflammation is still under debate (120,230). Given the increasing evidence that viruses are a major cause of acute exacerbation of asthma, the cytotoxic and potent antiviral properties of inducible NO may be beneficial (120). The interaction of NO with the transcription factor NF- $\kappa$ B, which is activated by diverse inflammatory stimuli, is causally linked to respiratory cell inflammation and pulmonary disease, but has not been demonstrated unambiguously (231). It should be noted that high concentrations of NO are capable of killing *Mycobacterium tuberculosis* and this may be significant for the control of infection in the lung (232).

Cerebrospinal fluid concentrations of the stable NO metabolite nitrite are elevated both in animal models of bacterial meningitis (BM) and in patients with the disease (233). In a model of BM, production of nitrite in the cerebrospinal fluid of rats correlated with elevated blood-brain barrier permeability, which suggests that NO contributes to the pathophysiology of BM (233). However, NO produced by iNOS may be beneficial as well. Recently, inhibition of iNOS, primarily localized to the cerebral vasculature and inflammatory cells in the subarachnoid and ventricular space, increased cortical hypoperfusion and ischemic neuronal injury in an infant rat model of meningitis caused by group B streptococci (234).

One of the primary functions of the inflammatory response is to heal wounded tissue. Healing commences soon after injury, while acute inflammation is still in full swing. Aseptic wounding induces iNOS, which may modulate wound healing (235); however, definitive proof of this concept requires wound-healing

studies in iNOS null mice. Our group showed a delay in closure of excisional wounds in iNOS-deficient mice, compared with wild-type mice. This defect in healing of excisional wounds could be quantitatively corrected by a single topical administration of an adenoviral vector expressing iNOS cDNA (236). Interestingly, the cytokine most associated with wound healing, TGF- $\beta$ 1, may be the most potent suppressor of iNOS (237). The recent finding that exposure to NO of cells, which express latent TGF- $\beta$ 1, could lead to the activation of this cytokine (35). This raises the intriguing suggestion that one of the roles of iNOS in wound healing is to modulate TGF- $\beta$ 1. Most importantly, these findings suggest caution with the use of iNOS inhibitors in settings that require appropriate wound healing.

## Conclusions

It is now clear that NO cannot be rigidly catalogued as either an anti-inflammatory or a pro-inflammatory molecule, but it can be considered a true inflammatory mediator. Although all three NOS isoforms are involved to a greater or lesser extent in the course of inflammation, the role of iNOS appears to be dominant. Inducible, high-level NO production mediates a number of inflammatory and infectious diseases by acting both as a direct effector and as a regulator of other effector pathways. The dichotomous role of NO in inflammation, often referred to as the NO paradox, is based mainly on the conflicting data showing the effects of NOS inhibitors of varying selectivity in different animal models. In addition, the use of iNOS null animals for exploring the role of NO further reinforces this view and clearly demonstrates that caution should be taken when extrapolating experimental results to possible therapeutic benefits. For example, the basal upregulation of heat shock protein 72 in iNOS null mice, rather than the inhibition of inducible NO production, is implicated in their protection from renal ischemia-reperfusion injury (238). Finally, the spectrum of activity of NO itself versus that of the reaction products of NO present under physiological and pathological conditions (10) may help account for this seeming paradox.

The expression of iNOS is reported in a variety of diseases. The level of iNOS expression and high output NO formation trigger short- and long-term effects that may be either bene-

ficial or deleterious, and ultimately depend on five factors:

- (1) the existence of additional metabolic pathways that provide the iNOS with substrate and cofactors;
- (2) the effect of other pathways that influence or affect the enzyme induction and activity;
- (3) the molecular targets with which NO and NO-derived species interact;
- (4) local factors, such as the cellular redox state; and
- (5) the presence and concentration of endogenous defense and antioxidant/scavenging mechanisms.

Administration of NO donors (in hypertension, angina, atherosclerosis, and gastrointestinal and genitourinary disorders), inhalation of NO gas (in chronic pulmonary hypertension or adult respiratory distress syndrome), or increased intake of L-arginine (in atherosclerosis) may be suitable therapies for disease states in which impaired NO production appears to exacerbate inflammation. The biggest challenge, therefore, is to develop strategies that target the cytotoxic and damaging actions of NO/RNOS without interfering with essential protective functions. Besides selectively inhibiting iNOS, a number of other therapeutic strategies are conceivable in order to alleviate the deleterious effects of excessive NO formation. These alternative therapies involve scavenging of NO/RNOS, and/or inhibition of metabolic pathways triggered by these molecules. The advantage of preserving the beneficial effects of iNOS also needs to be considered when implementing any therapeutic approach. Counteracting vascular hyper-responsiveness to endogenous vasoconstrictor agonists in septic shock, or inducing cardiac protection against ischemia-reperfusion injury are examples of such beneficial effects of iNOS. Only the identification of the roles of NO and of the cells that produce it, as well as the more complete elucidation of the mechanisms that regulate its cellular production in inflammation, will help in the development of therapeutic applications for both acute and chronic inflammatory diseases.

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