



Nitric Oxide and Infection: Another View

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Nitric oxide (NO) has been nicknamed “murderer” and “mediator” because it has toxic and signaling properties. We review these two aspects of NO synthesis from the perspective of the clinical infectious disease specialist by considering the potential of NO as an endothelium-derived relaxing factor (EDRF) in inflammation and sepsis and its potential as an antimicrobial system. We deviate from observations in recent authoritative reviews and point to important species differences that make it unlikely that NO serves as an EDRF mediating inflammatory vasodilatation in humans or that NO synthesized by human phagocytes has an antimicrobial function. We propose that in humans, NO synthesis is more confined and compartmentalized than in certain other animal species, and therefore, unwelcome toxicity, vasodilatation, or disturbance of paracrine signaling mechanisms (i.e., modulation of phagocytic cell functions) are avoided during inflammation.

Nitric oxide (NO) has received much attention over the last few years and was named molecule of the year in 1992 [1]. The recent scientific literature is replete with articles on the many aspects of biological and pathological processes involving NO (reviewed in [2–4]), suggesting at times that NO might be a jack-of-all-trades. Two aspects of the generation of NO are of particular interest to infectious disease specialists: (1) the potential role of NO as a cytotoxic molecule contributing to the antimicrobial armature of phagocytes and (2) the role of NO as a vasodilator of inflammation and mediator of septic shock. We outline our view on the role of NO in infectious processes in the human system. Our view deviates in some aspects from recent authoritative analyses [2–4] and takes into account evolutionary adaptations favorable to a utilization of this highly reactive and toxic molecule, nicknamed “murderer” [3], as a mediator.

NO is produced in human and laboratory animals by NO synthases (NOS). NOS isoenzymes [5] have been categorized into inducible and constitutive isoforms (table 1). However, it is apparent that activity of inducible NOS (iNOS) is also constitutively present in many tissues, and that a constitutively present activity of iNOS is further upregulated by enzyme-inducing signals in these cell types [6–8]. It has also become clear that the expression and activity of the constitutive NOS (cNOS) are modulated as well [9].

NO as Antimicrobial Product of Mononuclear Phagocytes

NO derived in mononuclear phagocytes by a high-output iNOS from L-arginine has been proposed to have antibacterial,

antifungal, and antiprotozoal activity [2–4]. In animal macrophages, notably murine macrophages, the pathway of NO synthesis and its regulation have been well characterized (reviewed in [10]). This includes consumption of the substrates L-arginine and O₂, production of the co-product L-citrulline, and the need of cofactors reduced nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), and tetrahydrobiopterin (BH₄). Most studies regarding the role of NO formation, including its role as an antimicrobial product, depend on quantitation of nitrite, an oxidation product formed spontaneously from NO in biological systems. The main reason for this is that nitrite can be measured by a convenient and technically simple test that can be performed in any laboratory. However, there are many potential pitfalls in correlating nitrite concentrations to NO production (figure 1, table 2). Artificial accumulation of nitrite by an unknown mechanism occurs in culture media incubated for 7 or more days without ever changing the medium. Nitrite formation is independent of the type of medium, the presence of living cells, or the presence of chemicals like sodium azide, and is significantly higher in the presence of IL-4. In media incubated at room temperature without CO₂, nitrite forms even more readily (our unpublished observation).

To demonstrate convincingly the production of NO, it is necessary either to use cumbersome assays measuring NO by chemoluminescence or to show that L-arginine is converted into L-citrulline along with nitrite production [11]. To date there is no convincing evidence that human phagocytes produce NO in amounts that could account for antimicrobial activity. Most, if not all, of the discrepancies in the scientific literature in regard to NO production by human phagocytes ensue from the confinement of these studies to the measuring of nitrite in biological systems. In addition, as of this writing no single laboratory has reported (based on stringent biochemical methods) that human phagocytes dispose of high-output NOS activity. Studies depending on L-arginine analogues used as “inhibitors” have also not been helpful in establishing a role for NOS

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Table 1. Tissue distribution of nitric oxide synthases in animal models.

Cellular source and activator(s)	cNOS	iNOS
Cellular source	Endothelium*	Endothelium [†]
	Neurons*	Hepatocytes*
	Epithelial cells*	Macrophages [†]
	Astrocytes*	Smooth muscle cells*
	Neutrophils [‡]	Carcinoma cell lines*
	Thrombocytes [‡]	Neutrophils [†]
		Islet cells [†]
		Mesangium cells*
		Cardiomyocytes [†]
		Fibroblasts [†]
		Bronchial epithelium*
Activators	Calcium ionophore Excitatory amino acids Acetylcholine, bradykinin	IFN- γ , LPS, in combinations: TNF- α , IL-1, IFN- α

NOTE. cNOS = constitutive nitric oxide synthase; iNOS = inducible nitric oxide synthase; LPS = lipopolysaccharide.

- * In animal and human cells.
- [†] In animal but not in human cells.
- [‡] Not defined in human cells.

as a killing system of human phagocytes. In such experiments, complex biological systems such as macrophages and target cells were exposed for prolonged times to these amino acid analogues; no appropriate control experiments were performed. In particular, the potential effects of these antimetabolites on protein synthesis of phagocytes or target cells and, above all, an effect on the growth phase of microorganisms that critically affects susceptibility to killing [12] were not addressed in such studies.

On the basis of these reflections, it cannot be overemphasized that, in contrast with animal studies [10] or studies on human hepatocytes [13], scientists have failed to demonstrate, by biochemical methods other than mere nitrite measurement, high-output iNOS activity in human mononuclear phagocytes, including tissue macrophages and microglia [11, 14, 15], as well as in neutrophils [16]. Another basic issue related to this is the fact that there are fundamental biochemical differences between human mononuclear phagocytes and phagocytes from certain animal species. Human mononuclear phagocytes lack relevant arginase or urea cycle activity, and therefore, they do not metabolize L-arginine by this pathway [8, 11]. It has also been shown that human mononuclear phagocytes lack the enzyme systems required for the synthesis of the BH₄, an essential cofactor required for NO synthesis (table 3). It should be incidentally noted that exogenous BH₄ can not support NO synthesis in human macrophages [11].

iNOS has been cloned, and the regulation of its expression has been studied in human, murine, and rat mononuclear phagocytes by northern analysis, which permitted quantitative assess-

ment of specific cellular mRNA levels. While lipopolysaccharide (LPS) and IFN- γ were able to induce within hours long-lasting synthesis of iNOS mRNA in phagocytes from rats and mice, no iNOS mRNA was detectable by this method in stimulated and unstimulated human phagocytes [6]. When a much more sensitive reverse-transcriptase PCR (RT-PCR) method was used, minute amounts of iNOS mRNA were transiently detected upon stimulation with LPS/IFN- γ in human peripheral blood mononuclear cells that had been purified of monocytes (but nevertheless contained 1%–2% contaminating cells of other types). It is noteworthy that no NOS activity was detectable in these experiments. The minute amounts of iNOS mRNA detected cannot be weighed against amounts found in mononuclear phagocytes from mice or rats [6]. In-depth studies of this phenomenon indicate that minute amounts of iNOS message can be detected by RT-PCR in apoptotic human mononuclear phagocytes, but that this message is not translated into a functional NOS enzyme [17, 18]. These observations confirm by molecular methods the results of antecedent biochemical studies: human mononuclear phagocytes do not transcribe or translate iNOS message at a level that could be compared with that in phagocytes from mice or rats. Genomic analyses that so far show similarities in the promoter regions of the rodent and human iNOS gene, particularly in respect to IFN- γ and LPS-dependent regions, cannot explain these important species differences [19, 20]. The similarities in LPS and IFN- γ -dependent promoter regions of the NOS gene in humans and mice also speak somewhat against the requirement for occult cytokine

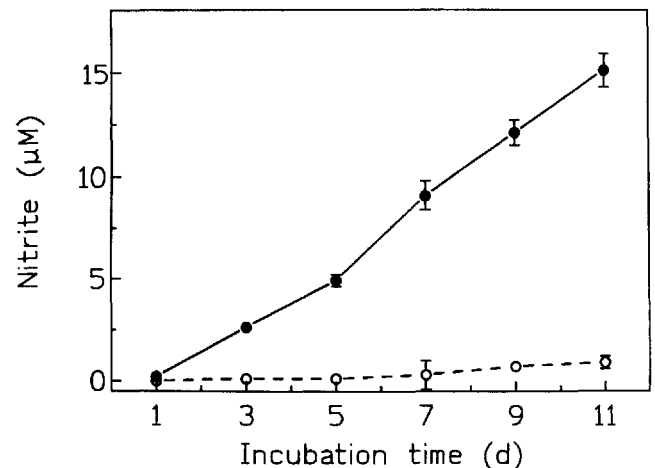


Figure 1. Spontaneous formation of nitrite in cell culture medium (RPMI 1640) supplemented with 5% fetal bovine serum, human recombinant IL-4 (200 U/mL), and human recombinant IFN- γ (500 U/mL). Cell-free medium was incubated (without changing) at 37°C, 5% CO₂ for the times indicated (●). In the absence of IL-4/IFN- γ , nitrite accumulation was minimal (○). This reproducible phenomenon was also seen with other cell culture media supplemented accordingly. None of the supplements contained sodium azide. Nitrite concentration = mean \pm SD from triplicate wells. Medium with and without supplements, $P < .01$ for all values ≥ 3 days of incubation (Student's t -test).

Table 2. Postulated pathways for nitric oxide production and appropriate experimental assessment.

Pathway	Experimental assay
Nitrite formed spontaneously in cell-free medium	Set up cell-free controls accordingly with identical supplements to media; measure L-citrulline production.
Nitrite formed by ROIs	Use phagocytes defective in ROI generation; use ROI inhibitors; measure L-citrulline; use substrate analogues for control.
Nitrite formed by microbial terminal oxidases or microbial nitric oxide synthase	Measure L-citrulline; control for metabolically active microorganisms.
Nitrite formed by tumor cell target in cytotoxicity experiments with macrophages	Use target cells without nitric oxide synthase that can be induced during coculture by cytokines released from macrophages.
Undesired antimetabolic effects of L-arginine analogues	Do not depend on analogues; use substrate antagonist only briefly during killing; check for effects on protein synthesis and other cell functions of phagocytes and target cells.

NOTE. ROI = reactive oxygen intermediate.

Table 3. Metabolic differences between murine and human macrophages concerning the L-arginine/nitric oxide pathway.

Parameter	Murine macrophages	Human macrophages
L-arginine consumption	600–800 $\mu\text{mol}/10^6$ cells	10–20 $\mu\text{mol}/10^6$ cells
Arginase activity	Yes	No
Ornithine production	500–700 $\mu\text{mol}/10^6$ cells	<0.1 $\mu\text{mol}/10^6$ cells
Urea production	800–1,100 $\mu\text{mol}/10^6$ cells	<0.1 $\mu\text{mol}/10^6$ cells
Nitric oxide synthase activity	1.4–2.2 $\mu\text{mol}/(\text{h}\cdot 10^6)$ cells	<0.3 $\mu\text{mol}/(\text{h}\cdot 10^6)$ cells
Citrulline production	60 $\mu\text{mol}/10^6$ cells	<0.1 $\mu\text{mol}/10^6$ cells
Cytosolic BH ₄	10–90 pmol/mg protein	<0.03 pmol/mg protein

NOTE. Table was adapted from references [8, 11]. BH₄ = tetrahydrobiopterin.

brews that would instruct viable human macrophages to express iNOS, a concept recommended by those unwilling to accept the undeniable species differences.

Taken together, these findings show that observations made on the role of iNOS in the antimicrobial or antitumoral function of phagocytes from mice, rats, or other species can not be simply transferred to human biology. To date, there is no indication that an inducible high-output NOS is antimicrobially operative in human phagocytes (or, incidentally, in rabbit macrophages) [11].

We have previously proposed that one reason for these species differences might be redundancy of phagocytic killing systems that would allow for species variability [11]. Because species differences regarding L-arginine metabolism of phagocytes are not restricted to iNOS but include arginase and other enzymes of the urea cycle (table 3), these disparities must perhaps be seen in a broader context that includes other metabolic pathways. Finally, the question occurs as to whether it was advantageous, in an evolutionary sense, to limit the synthesis of NO, which might not only have antimicrobial functions but could also become injurious to the host, mediating disastrous host responses such as vasodilatation during septic shock.

The Role of NO in Inflammatory Vasodilatation

In view of the major species differences in the regulation and expression of iNOS in phagocytes, the question arises as to whether comparable differences exist in NOSs of blood vessels. The role of NO in the control of the vascular tone has recently been extensively reviewed [2–4]. There is solid evidence that NO is an endothelium-derived relaxing factor (EDRF) mediating physiological vasodilatation in animals and humans. In addition, vasodilatation during pathological states such as sepsis and inflammation appears to be mediated by a nitrovasodilator system. In several animal species, iNOS activity is induced by inflammatory mediators in endothelial cells, an activity that is comparable to that of the high-output iNOS in murine macrophages [2]. It is therefore conceivable that NO also plays an important role as an inflammatory EDRF in these species [2–4]. Comparable to observations made in macrophages, however, no high-output iNOS activity can be induced in human endothelial cells by LPS, IFN- γ , TNF- α , or IL-1 [21, 22], mediators that induce high-output NOS activity in macrophages and endothelial cells from rats, mice, and cattle or in human hepatocytes [13]. Furthermore, cNOS activity de-

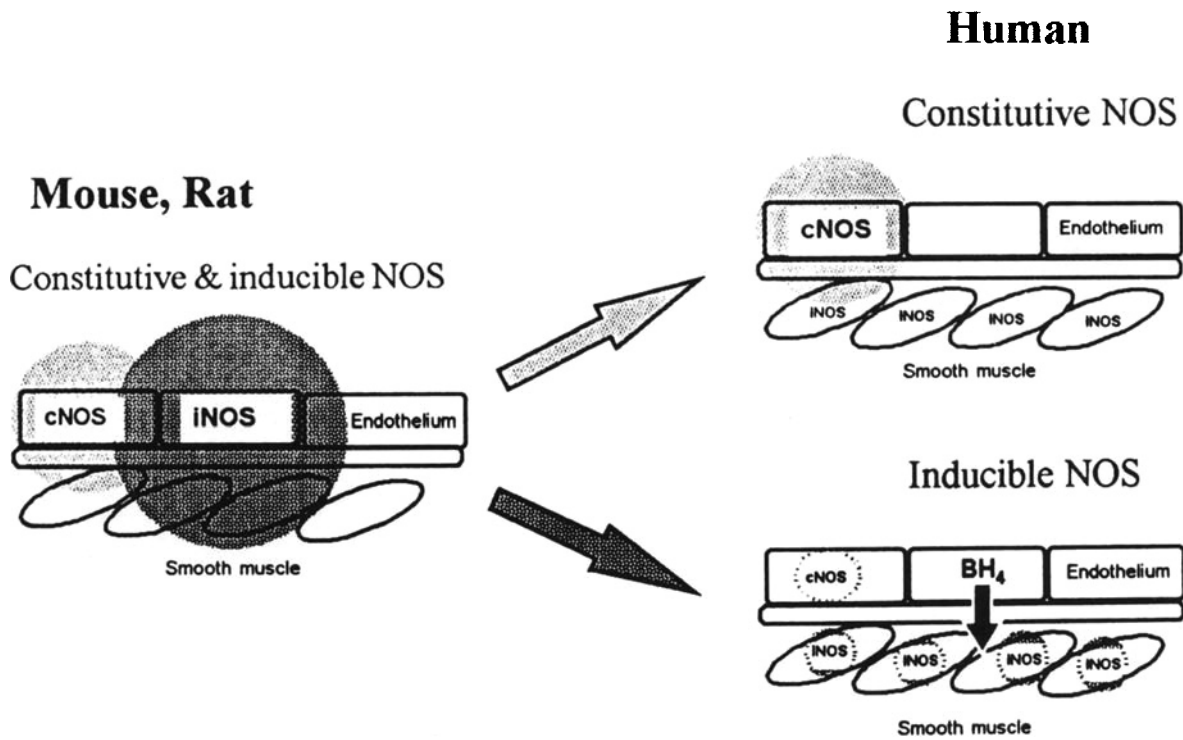


Figure 2. Evolutionary compartmentalization of vascular nitric oxide (NO) synthesis. In mice and rats, endothelial inducible nitric oxide synthase (iNOS) present along with the constitutive enzyme (cNOS) has the potential to secrete NO in amounts comparable to toxic amounts produced by rodent macrophages. In contrast, by compartmentalization of NO synthesis in the human vascular system into constitutive endothelial cell (physiological, *light gray arrow*) and inducible smooth muscle cell (inflammatory, *dark gray arrow*) isoenzymes, the secretion of large amounts of potentially toxic and randomly diffusing NO synthesized during inflammatory response can be avoided. We propose that endothelial cells regulate NO synthesis in smooth muscle cells through tetrahydrobiopterin (BH₄) to conserve inflammatory endothelium-derived relaxing factor function through the nitrovasodilator system, *Shaded areas*: correspond to random NO diffusion.

creases in endothelial cells under the influence of inflammatory stimuli, and mRNA for cNOS accordingly decreases after exposure to IFN- γ and/or LPS without *de novo* expression of iNOS mRNA in human endothelial cells [21]. Therefore, NO, in contrast to its role in physiology, appears not to be an endothelial mediator (EDRF) of inflammatory vasodilatation in the human system. The question then arises as to where the production of NO occurs that is responsible for the inflammatory vasodilatation attributed to the nitrovasodilator system in humans. Because inflammatory mediators induce iNOS mRNA synthesis in human vascular smooth muscle cells (SMCs), it appears plausible that inflammatory vasodilatation is prompted in humans by NO synthesized within the SMCs themselves. NO activates soluble cytosolic guanylate cyclase in SMCs, resulting in an increase of the intracellular cyclic guanosine monophosphate (cGMP) level, which in turn mediates muscle relaxation [23, 24]. Synthesis of NO at the very site of its action within SMCs appears advantageous over a more distant synthesis in endothelial cells. This advantage might be particularly important in relation to inflammatory vasodilatation caused by high-output iNOS, which is apt to produce large amounts of NO. Randomly diffusing NO produced by an endo-

thelial iNOS could lead to considerable tissue damage (figure 2).

All observations made in human cell systems point out that NO is not the inflammatory EDRF. How then is vasodilatation controlled under inflammatory conditions? As mentioned earlier, inflammatory stimuli such as LPS and IFN- γ induce synthesis of iNOS in vascular SMCs, but endothelial factors must also be considered for the regulation of inflammatory vasodilatation. We have recently shown that BH₄, an obligatory cofactor of NOS, is a highly regulated secretory product of human endothelial cells [25]. In cell culture experiments, exogenous BH₄ has been found to have regulatory effects on NO synthesis and to augment iNOS activity as a limiting factor [8, 23]. Upon inflammatory stimuli, BH₄ synthesis precedes an increase in iNOS activity by several hours. In addition, simultaneous inhibition of BH₄ synthesis prevents increased iNOS activity [26]. In isolated rat aorta it has been shown that BH₄ induces a marked and long-lasting vasodilatation [27]. These findings made us consider the possibility that BH₄ is an EDRF mediating inflammatory vasodilatation in the human vascular system. BH₄ secretion by human endothelial cells is markedly regulated by a factor of 70 by activating and deactivating cytokines [25].

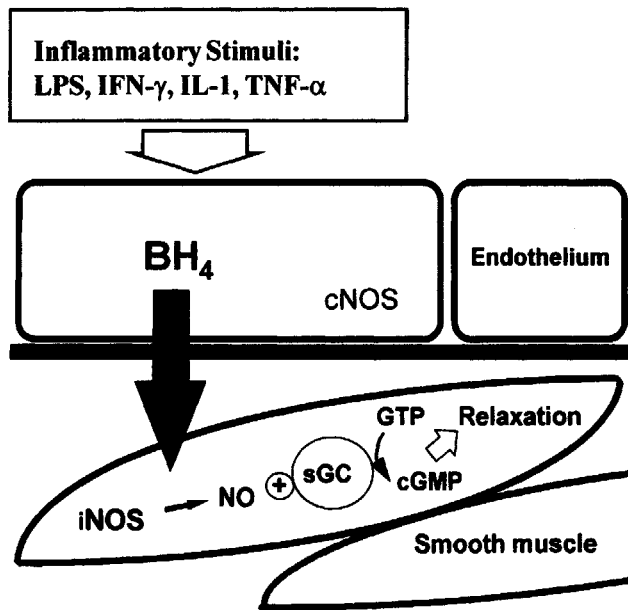


Figure 3. Proposal for regulation of nitric oxide (NO) synthesis in smooth muscle cells (SMCs) by endothelial tetrahydrobiopterin (BH₄) functioning as an endothelium-derived relaxing factor. While inflammatory stimuli downregulate the expression of constitutive NO synthase (cNOS) in human vascular endothelial cells, they concomitantly induce in these cells the synthesis and vectorial secretion of BH₄ in the direction of SMCs. In parallel, inflammatory stimuli upregulate expression of inducible NO synthase (iNOS) in human vascular SMCs. BH₄ is a limiting factor for NO synthesis by iNOS in inflammatory stimulated SMCs. Endothelium-derived BH₄ acts as first messenger modulating NO production in the SMCs, which as second messenger activates soluble guanylate cyclase (sGC) to synthesize cyclic guanosine monophosphate (cGMP), which mediates relaxation as third messenger.

In addition, endothelial cells secrete BH₄ vectorially into the direction of vascular SMCs [23]. Furthermore, we found that intraarterial infusion of BH₄ results in a local vasodilatation that is accompanied by local consumption of L-arginine and production of cGMP [23], strong indications that BH₄ can act as a mediator of vasodilatation through the nitrovasodilator pathway. In this way, BH₄ acts as first messenger controlling NO production in SMCs, which as second messenger controls the synthesis of cGMP, which regulates vasodilatation as third messenger. Based on these observations we propose that not NO but BH₄ is an EDRF in the human vascular system inducing inflammatory vasodilatation (figure 3).

These details are of interest because BH₄ synthesis is amenable to pharmacological interventions that are aimed at reducing inflammatory vasodilatation. Accordingly, inhibition of BH₄ synthesis by coumarins in murine endothelial cells results in a reduction of NO synthesis [26]. Furthermore, there are indications that coumarins can protect animals from experimental septic shock by decreasing NO synthesis [28]. This approach does not necessarily affect the regulation of the vascular tone by NO synthesized by endothelial cNOS under physiological

conditions. In septic shock, however, the selective inhibition of BH₄ synthesis might be advantageous over a global inhibition of NO synthesis by L-arginine analogues affecting both iNOS and cNOS, which has a detrimental outcome in a canine model of septic shock [29].

Conclusions and Possible Significance of Evolutionary Variations in the Tissue Localization of iNOS

In conclusion, we propose that NO synthesis in humans is more restricted than in other mammalian species such as the rat and mouse and that not all human tissues dispose of iNOS activity. In humans, prevention of an unhindered redundant expression of iNOS in phagocytes circumvents undesired vasodilatory signaling and toxic effects. Accordingly, compartmentalization of iNOS activity in human vessel walls with restriction of NO synthesis to SMCs during inflammation could be advantageous because it would avoid autotoxicity of NO. Furthermore, restricted compartmentalized NO synthesis may permit, even during inflammation, paracrine signaling that modulates phagocyte functions [30]. Taken together, the two observations on iNOS in human phagocytes and the vascular system could be viewed as an evolutionary refinement in the use of this intriguing molecule, which can be both a murderer and an essential mediator of biological responses.

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