Nitric Oxide and Peroxynitrite Production in Ocular Inflammation

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Recent studies have implicated nitric oxide and peroxynitrite in the pathogenesis of many diseases, such as septic shock, arthritis, lung disease, and atherosclerosis. Nitric oxide (•NO) exerts many diverse effects on vascular tone, affecting neurotransmission and cellular cytotoxicity/communication. Our laboratory and others have documented a proinflammatory role for •NO in ocular inflammation. Uveitis, which is an inflammation of the highly vascular uveal tract in the eye, is a debilitating condition that can lead to visual impairment and blindness. It is characterized by acute, recurrent, or persistent inflammation with disruption of the blood–aqueous barrier and is accompanied by protein leakage and leukocyte infiltration into the aqueous humor and anterior chamber. Systemic injection of endotoxin into mice and rats, or intraocular injection of endotoxin into mice, rats, and rabbits induces acute uveitis, which clinically and histologically resembles acute anterior uveitis in humans. These models facilitate the study of pathogenic mechanisms that contribute to ocular inflammation. In addition to •NO, superoxide anion radicals ($O_2^{\bullet -}$), and peroxynitrite ($ONOO^-$), the products of the reaction between •NO and $O_2^{\bullet -}$, are also implicated in uveitis. The role of peroxynitrite in ocular inflammation is still largely unknown. Characterization of the roles of these important uveitic mediators in the ocular inflammatory response will provide information critical to the understanding of the pathogenesis of intraocular inflammation so that more effective therapeutic intervention(s) can be developed. — *Environ Health Perspect* 106(Suppl 5):1145–1149 (1998). *http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-5/1145-1149allen/abstract.html*

Key words: nitric oxide, peroxynitrite, uveitis, SIN-1, SOD, inflammation, NOS inhibitors

Introduction

Intense research has focused on the role of nitric oxide (•NO) and peroxynitrite ($ONOO^-$) in physiologic and pathophysiologic processes. •NO is beneficial and necessary for normal physiologic processes such as vasodilation (*1*), platelet functions (*2*), and neurotransmission (*3,4*). In contrast, higher concentrations of •NO are thought to be cytotoxic and may contribute to cell injury or inflammation in many disease states such as arthritis (*5*), lung disease (*6*), and atherosclerosis (*7*). The detrimental toxic effects of •NO may be due to the formation of $ONOO^-$ from the reaction between •NO and superoxide anion radical ($O_2^{\bullet -}$). Recent studies suggest that $ONOO^-$ may also have both beneficial and detrimental effects (*8*).

The role of •NO in the eye has been extensively investigated. The contribution of this regulatory molecule to ocular physiology is emerging, but additional attention must be directed toward the understanding of the pathology of eye diseases. To this end, animal models of uveitis have been extensively used to try to elucidate the complex cascade of events leading to uveitis. Uveitis involves the recruitment of leukocytes into the uvea and the disruption of the blood barrier, resulting in protein extravasation and cellular infiltration into the aqueous humor and anterior chamber (*9,10*). Studies from animal models of uveitis and human patients with uveitis implicate reactive oxygen/nitrogen radicals in this inflammatory process. In the studies detailed below, the importance of the reactive radicals in the initiation and development of ocular inflammation will be discussed.

Nitric Oxide

Nitric oxide is a small, membrane-permeable free radical synthesized from the terminal guanidino nitrogen of L-arginine by the enzyme •NO synthase (NOS) (*11*). NOS exists in three isoforms and is differentially expressed in many different cell types (*12,13*). The different isoforms are transcribed from three distinct genes and catalyze the oxidation of L-arginine to L-citrulline (*14,15*). Required cofactors include NADPH, flavin adenine dinucleotide, flavin mononucleotide, calmodulin, heme, and tetrahydrobiopterin. The three NOS isoforms differ in calcium dependence, tissue distribution, amino acid sequence, regulation of expression, and function (*16*).

Endothelial NOS (eNOS or NOS3; 135 kDa) and neuronal NOS (nNOS or NOS1; 150–160 kDa) are constitutively expressed, require calcium, and are collectively referred to as constitutive NOS (cNOS) (*3,17,18*). Small amounts of •NO are produced by these enzymes. •NO generated from the eNOS pathway activates soluble guanylyl cyclase to produce the intracellular signaling molecule cGMP, which functions to relax vascular smooth muscle cells by decreasing intracellular calcium (*18,19*). •NO produced by nNOS functions as a neurotransmitter in the nervous system (*3,4*).

A third isoform, an inducible NOS (iNOS or NOS2; 130 kDa), usually does not exist under normal conditions and is expressed in response to certain cytokines

This paper is based on a presentation at the Second International Meeting on Oxygen/Nitrogen Radicals and Cellular Injury held 7–10 September 1997 in Durham, North Carolina. Manuscript received at *EHP* 5 February 1998; accepted 17 June 1998.

The authors thank M.C. McGahan, L. Fleisher, and K. Adler for helpful discussions and collaborations during the early aspects of this project. Also, the authors are grateful to A. Bond and J. Ferrell for expert technical assistance during the course of these experimental studies. This work was supported by National Institutes of Health grants EY11364-01, 5P30EY05722; funds from the State of North Carolina, and APEX Bioscience, Inc.

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Abbreviations used: AF, aqueous flare; AG, aminoguanidine; cNOS, constitutive nitric oxide synthase; EIU, endotoxin-induced uveitis; D-NAME, N^G-nitro-D-arginine methyle ester; eNOS or NOS3, endothelial nitric oxide synthase; IL, interleukin; iNOS, inducible nitric oxide synthase; L-NAME, N^G-nitro-L-arginine methyl ester; LPS, lipopolysaccharide; nNOS or NOS1, neuronal nitric oxide synthase; •NO, nitric oxide; NOS, nitric oxide synthase; $ONOO^-$, peroxynitrite; $O_2^{\bullet -}$, superoxide anion radical; SIN-1, 3-morpholinosydnonimine hydrochloride; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SOD, superoxide dismutase; TNF, tumor necrosis factor.

(interleukin [IL]-1β, tumor necrosis factor [TNF]-α) and bacterial products (lipopolysaccharide [LPS]) (*13,20,21*). Many cell types have the capacity to express iNOS after exposure to certain immunologic or inflammatory stimuli (*22–29*). High concentrations of •NO generated by this pathway are sustained for prolonged periods of time *in vitro* and *in vivo* (*13,30*). iNOS expression is delayed 2 to 8 hr after exposure to inducing cytokines; the delay is necessitated by the synthesis of essential cofactors (*31*). Elevated levels of •NO generated by the iNOS are thought to be involved in many inflammatory conditions (*32–35*).

The production of •NO can be inhibited by analogs of L-arginine, which compete with L-arginine for the active site of NOS (*12,36–38*). Nonamino acid compounds also inhibit NOS activity (*39*). Furthermore, inhibition of •NO can be achieved by limiting necessary cofactors of NOS activity and inhibiting signal transduction and transcription of NOS (*39*). NOS inhibitors exhibit different specificities for the NOS isoforms (*22,39*). For example, aminoguanidine (AG) is a more selective iNOS inhibitor (*40,41*) and N^G-monomethyl-L-arginine and N^G-nitro-L-arginine methyl ester (L-NAME) inhibit both cNOS and iNOS (*22*). More selective NOS inhibitors are needed to dissect the involvement of the different NOS isoforms in both physiologic and pathophysiologic processes.

Peroxynitrite

Peroxynitrite is a potent oxidant that is generated from •NO and $O_2^{\bullet-}$ (*42*). Under pathologic conditions such as ischemia–reperfusion injury, inflammation, and sepsis, elevated levels of both •NO and $O_2^{\bullet-}$ are produced by tissues. Because $O_2^{\bullet-}$ reacts with •NO at a faster rate than with superoxide dismutase (SOD), a scavenger of $O_2^{\bullet-}$, $ONOO^-$ may be formed at high yields and contribute to the pathology. $ONOO^-$ is also implicated in the pathogenesis of rheumatoid arthritis, shock associated with endotoxemia, silica-induced lung injury, neurotoxicity, endothelial injury, and colonic inflammation (*43–45*). $ONOO^-$ directly and rapidly oxidizes sulfhydryl groups and initiates lipid peroxidation (*46*). Alternatively, $ONOO^-$ can nitrate phenolic rings of tyrosine residues of proteins, leading to the formation of nitrotyrosine, which is easily detected with antinitrotyrosine antibodies (*47*). Thus, the presence of nitrotyrosine can serve as a $ONOO^-$ "footprint."

Just as is the case for •NO, other studies suggest that $ONOO^-$ can mediate physiologic processes that are beneficial. $ONOO^-$ produces vascular relaxation in isolated coronary (*48*) and pulmonary arteries (*49*). It also inhibits platelet aggregation (*50*). An important study by Lefer et al. (*8*) provides evidence that $ONOO^-$, at nanomolar concentrations, significantly inhibits leukocyte–endothelial interactions both *in vitro* and *in vivo* by inhibiting the upregulation of P-selectin. In addition, the study showed that polymorphonuclear accumulation in ischemic–reperfused rat hearts was reduced by nanomolar concentrations of $ONOO^-$. The differences in beneficial versus detrimental effects of $ONOO^-$ may be, at least in part, concentration dependent, and underscore the complex biochemistry of •NO and $ONOO^-$.

•NO and $ONOO^-$ in the Eye

The function of •NO in the eye has been extensively studied, from physiologic and pathophysiologic functions to therapeutic potential (*51*). All three NOS isoforms have been identified in different parts of the eye, either with NADPH-diaphorase staining or specific anti-NOS antibodies. There are multiple sites of action for the signaling properties of •NO produced by NOS1 and 3 isoforms. These NOS isoforms have been detected in the retina and in vascular endothelium of the anterior segment and choroid, in conjunctiva, and in ciliary muscle and trabecular meshwork. This suggests a therapeutic potential for the use of •NO donors (nitrovasodilators) in controlling retinal blood flow, aqueous humor dynamics, and regulation of intraocular pressure. Behar-Cohen et al. (*52*) evaluated the effect of intraocular administration of •NO donors in the rabbit eye and observed a drastic decrease in intraocular pressure, with no inflammatory or toxic effects on ocular tissues. The NOS2 isoform produces larger amounts of •NO, which contributes to cytotoxicity, and has been identified in the retina (pigmented epithelium, pericytes, and Muller cells) and iris–ciliary body. During uveitis, infiltrating polymorphonuclear and mononuclear cells express NOS2, probably after stimulation by uveitic cytokines such as IL-1β and TNF-α.

In an experimental guinea pig model of allergic conjunctivitis, •NO was shown to mediate increased conjunctival blood flow and hyperpermeability of sensitized guinea pigs after exposure to the antigen (48 of 80), or to histamine. Increased levels of nitrite were detected in lavage fluid 30 min after challenge, along with conjunctivitis, edema formation, and hyperpermeability. These inflammatory parameters were inhibited with pretreatment with L-NAME or AG (*53,54*). Neurotoxicity of NO in the retina was investigated in rabbits by intravitreal injection of the •NO donor *S*-nitroso-*N*-acetylpenicillamine (*55*). Retinal toxicity of •NO was shown both functionally and histologically, suggesting that •NO may play a role in retinal ischemia or in degenerative diseases. As in other physiologic and pathologic situations, the protective and detrimental effects of •NO and $ONOO^-$ are complex and require further investigation. The ability of •NO and $ONOO^-$ to lower intraocular pressure and increase retinal blood flow suggests a beneficial use in treatment of certain ocular situations such as glaucoma, but the potential retinal toxicity associated with increased •NO should be considered and investigated further.

•NO and $ONOO^-$ in Experimental Uveitis

In an experimental autoimmune uveitis model, Wu et al. (*56*) demonstrated $ONOO^-$ formation in the photoreceptors, and to a lesser extent in the ganglion cell layers, nerve fiber cell layers, and blood vessels of the retina. These sites of $ONOO^-$ production appear to correlate with the area of pathologic oxidation and may be a major factor in the degeneration of photoreceptors in this model.

Several investigations using the endotoxin-induced uveitis (EIU) mouse and rat models document the importance of •NO in ocular inflammation (*57–65*). Intraperitoneal injections of L-NAME, an inhibitor of both cNOS and iNOS, significantly decrease aqueous protein and cell numbers in the anterior chamber of EIU in rats (*60*). Tilton et al. (*59*) used the more specific iNOS inhibitor AG and demonstrated that the hemodynamic and vascular changes associated with EIU are in part due to increased •NO production. Others have shown increased iNOS mRNA in the iris–ciliary body in EIU by reverse transcriptase–polymerase chain reaction (*64*) and *in situ* hybridization (*66*). These studies conclude that •NO is an important mediator of uveitis and that inhibition of NOS decreases ocular inflammation.

The EIU model in rabbits is clinically and histopathologically similar to acute

anterior uveitis in humans (*9,10*). The acute inflammation is associated with vascular and permeability changes in the anterior chamber of the eye. Studies of the pathogenesis of uveitis from human uveitis and animal models of ocular inflammation have identified increased cytokine (IL-1β, TNF-α, and IL-6) levels in uveitic ocular fluids and tissues compared to normal fluids and tissues (*67–69*). In addition, prostaglandins and other mediators have been associated with uveitis. Studies using the EIU model in rabbits have documented a role for •NO and $ONOO^-$ in ocular inflammation (*65,70*).

Endotoxin-induced uveitis in rabbits is induced by a single intravitreal injection of endotoxin (10 ng; *Escherichia coli*) into one eye of New Zealand white male rabbits (2–3 kg) and the appropriate vehicle into the contralateral eye (*9,10*). Temporal assessment of uveitis is monitored by direct examination of the anterior segment with a slit-lamp biomicroscope. Evidence of disruption of ocular barriers (protein extravasation; "aqueous flare") is graded from 0 to 4, with 0 being normal and 4 being maximal light diffraction (*71*). Iridal hyperemia, a measure of iridal blood vessel dilation in the iris–ciliary body in addition to aqueous flare, is also graded (0–4). These more subjective observations are quantitated at the termination of the experiment by measuring aqueous protein levels and counting the number of aqueous inflammatory cells (*9,10*). The eyes are removed and processed for histologic/molecular analyses. Hematoxylin and eosin-stained sections of eye, removed 18 to 24 hr after endotoxin injection, show a predominantly polymorphonuclear infiltrate with some mononuclear cells and a proteinaceous exudate in the anterior chamber. Animals are used in accordance with the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research and approved by the North Carolina State University Institutional Animal Care and Use Committee.

In an attempt to dissect the importance and contribution of the different NOS isoforms in uveitis, the authors used the iNOS and cNOS (L-NAME; 100 mM and 10 mM) and iNOS (AG; 114 mM) inhibitors in EIU in rabbits (*65*). These and additional published studies underscore the importance of determining which NOS isoform(s) is important at various stages of the inflammatory process. Rabbits were intravitreally injected in one eye with endotoxin (10 ng) with and without inhibitor and with endotoxin-free saline (vehicle) in the contralateral eye. Uveitis in rabbits coinjected with endotoxin and L-NAME was almost completely abolished in a dose-dependent manner, whereas coinjection of endotoxin and D-NAME, N^G-nitro-D-arginine methylester, the inactive enantiomer, did not suppress uveitis. Co-injection of endotoxin and AG, a more selective inhibitor of iNOS, significantly suppressed all parameters of EIU and suggested the importance of iNOS in ocular inflammation. To determine if NOS inhibitors have an effect on established uveitis, L-NAME was injected 0.5 hr and 6 hr after endotoxin administration. L-NAME still inhibited uveitis when injected 0.5 hr after endotoxin. However, injection of L-NAME 6 hr after endotoxin actually exacerbated the inflammatory response. In contrast, injection of AG 6 hr after LPS did inhibit cellular infiltration and protein extravasation. Thus, the differential effects of NOS inhibitors with different specificities underscore the importance of the time of their administration in ameliorating ocular inflammation, and could have significant impact on our understanding of uveitis and possible therapeutic intervention.

Because $O_2^{\cdot-}$, which reacts with •NO to form $ONOO^-$, also plays a role in intraocular inflammation (*72*), we examined aqueous humor proteins for evidence of $ONOO^-$ formation by examining protein nitration using immunoblot analysis (*47*). Several immunoreactive bands were observed in the EIU aqueous humors, compared to a decreased number of bands of immunoreactivity in vehicle-injected aqueous humors. The immunoreactivity was eliminated by co-incubation of antibody with 10 mM nitrotyrosine. In addition, aqueous humors from eyes co-injected with inhibitors of NOS contained decreased levels of immunoreactivity. Sections of iris-ciliary processes from inflamed eyes stained with antinitrotyrosine antibody showed positive staining, primarily in the cellular infiltrate. Taken together, these results suggest that $ONOO^-$ may also be involved in uveitis. Thus, by inhibiting the formation of •NO, formation of the tissue-damaging $ONOO^-$ would also be reduced, and intraocular inflammation is attenuated.

Additional studies to assess the potential direct inflammatory effects of $ONOO^-$ in the eye were performed (*73*). Rabbits were injected intravitreally every 3 hr for 15 hr with 3-morpholinosydnonimine hydrochloride (SIN-1) (5 mM), which donates •NO and $O_2^{\cdot-}$ simultaneously and therefore $ONOO^-$ or SIN-1 + SOD (10 mM); contralateral eyes received the same volume and number of injections of vehicle. Coinjection of an excess of the $O_2^{\cdot-}$ scavenger SOD with SIN-1 results in only •NO formation and allows intravitreal effects of •NO and $ONOO^-$ to be independently evaluated. Eighteen hours after injection, rabbits injected with SIN-1 developed moderate iridal vasodilation and disruption of the blood–aqueous barrier, with some cellular infiltration into the aqueous humor. SIN-1 + SOD^- and vehicle-injected rabbits did not exhibit significant responses. These results suggest a direct role for $ONOO^-$ in uveitis. Characterization of these important uveitic mediators in the ocular inflammatory response will provide information critical to understanding the pathogenesis of intraocular inflammation.

Conclusions

Administration of NOS inhibitors with different isoform specificities at the onset or during EIU exerts differential effects on this inflammatory response, indicating that •NO is an important mediator of uveitis. We have demonstrated evidence of $ONOO^-$ formation in uveitic ocular fluids, and that $ONOO^-$ exhibits direct inflammatory effects in the eye. The studies described above underscore the importance of •NO and $ONOO^-$ in ocular physiology and pathophysiology. Development of even more selective NOS inhibitors will be necessary in order to distinguish the involvement of the different NOS isoforms, thus allowing for control of harmful, induced •NO production, while not affecting the physiologic actions of constitutive •NO release.

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