Role of Nitric Oxide Synthase Isozymes in Endotoxin-Induced Uveitis

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Purpose. The authors previously reported that in vitro treatment with Nω-nitro L-arginine (L-NNA), an inhibitor of nitric oxide synthase (NOS), reduces aqueous humor (AH) protein and cellular infiltration in endotoxin-induced uveitis in the rat eye. The objective of the current study was to determine the role(s) of respective major forms (constitutive and inducible) of NOS by comparing the effects of relatively selective inhibitors of these NOS isozymes.

Methods. Nω-nitro L-arginine (L-NNA), a relatively selective inhibitor for constitutive NOS (c-NOS), and N-iminoethyl L-ornithine (L-NIO), a more selective inhibitor for inducible NOS (i-NOS), were administered in vivo. Male Lewis rats were footpad injected with bacterial lipopolysaccharide (LPS, 200 μg) and were injected intraperitoneally at 0 hours, 6 hours, or both, after LPS injection with 10 mg of NIO, NNA, or saline as a control. Nitric oxide synthase activity in the ocular tissue and AH protein and cell content were determined at various times after treatment with LPS.

Results. After in vivo treatment, L-NIO was found to be a more potent inhibitor than L-NNA for ocular i-NOS (87% versus 43% inhibition), and L-NNA was more potent than L-NIO for ocular c-NOS (81% versus 39%). Two injections of L-NNA, one at time 0 and one 6 hours after LPS injection, inhibited the AH protein increase by 71%, but L-NIO did so by only 30%. L-NNA inhibited cellular infiltration by 86%, whereas L-NIO had no significant effect on cellular infiltration. A significant inhibition of cellular infiltration and AH protein increase also was observed with a single injection of 10 mg of L-NNA but not of L-NIO when the inhibitors were given simultaneously with LPS. Thus, reduction of uveitis symptoms correlates with the inhibition of c-NOS.

Conclusions. The constitutive form of NOS in ocular tissue, presumably in vascular endothelial cells, appears to play a critical role at the onset of the development of endotoxin-induced uveitis. Invest Ophthalmol Vis Sci. 1996;37:826-832.

Endotoxin-induced uveitis (EIU) is an experimental model of intraocular inflammation that is elicited by the administration of bacterial lipopolysaccharide (LPS).1,2 This uveitis model presents a rapid inflammatory reaction characterized by infiltration of polymorphonuclear cells and extravasation of plasma proteins into the anterior chamber of the eye, which peak at 24 hours after LPS injection. We previously reported3 that there is a significant increase in nitric oxide synthase (NOS) activity in ocular tissues during the course of this disease (peak at 9 hours after LPS injection); furthermore, an inhibitor of NOS, Nω-nitro L-arginine (L-NNA), significantly reduced the cellular infiltration in the anterior chamber and ameliorated protein increase in the aqueous humor (AH).

Nitric oxide synthase is subgrouped into constitutive and inducible forms.4-6 Constitutive NOS (c-NOS) is expressed constitutively in vascular endothelium and some neuronal cells, and its activity is calcium dependent. Inducible NOS (i-NOS) is expressed after stimulation with LPS, cytokines, or both, and it is regulated at the transcription level of the gene. Once induced, i-NOS, which is calcium independent, produces large amounts of NO for a longer period than
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c-NOS does. Inducible NOS is known to be induced in many types of cells, including polymorphonuclear leukocytes, and is thought to be involved in some pathologic conditions in which these types of cells are involved. Inducible NOS is known to be induced whereas no enhanced uptake of L-NNA was observed in leukocytes, and is thought to be involved in some pathologic conditions in which these cells are a major cause of inflammation and tissue damage. It has been reported that L-NNA is not as effective an inhibitor for i-NOS as for c-NOS. Yet, our previous observations showed that L-NNA not only reduced protein extravasation into the anterior chamber, it also blocked the entry of LPS-activated cells into the eye. This suggests that in EIU, not only i-NOS but also constitutive NOS (c-NOS) may participate in the disease course and that c-NOS may be involved even before the entry of activated cells into the eye. Hence, the questions addressed here are: Is NOS involved in the development of EIU at a stage earlier than cellular infiltration? If so, is the endothelial NOS (e-NOS), a constitutive NOS isozyme in endothelial–barrier cells, involved in the process of cellular infiltration?

In the current study, we used two NOS inhibitors, L-NNA and N-iminoethyl L-ornithine (L-NIO), to discriminate the two classes of enzyme. L-NNA is more selective for c-NOS than for i-NOS based on its reported IC50 value, and it can cause irreversible inactivation of c-NOS. In contrast, L-NIO has been shown to be a potent and selective irreversible inhibitor of i-NOS in phagocytes. Baydoun and Mann reported facilitated uptake of L-NIO by LPS-activated macrophages through the cationic transport system, whereas no enhanced uptake of L-NNA was observed by these cells. Based on these findings, L-NNA can be considered a potent inhibitor of c-NOS, and L-NIO can be considered a potent inhibitor of i-NOS in vivo. Therefore, we studied the effects of these agents on NOS activity, cellular infiltration, and protein extravasation in the ocular tissue and the selectivity for c-NOS in aortic endothelial cells and i-NOS in peritoneal phagocytes.

MATERIALS AND METHODS

Materials

Lipopolysaccharide (Salmonella minnesota) and nicotinamide-adenine dinucleotide phosphate were purchased from Sigma (St. Louis, MO). L-arginine and calmodulin were obtained from Wako Pure Chemicals (Osaka, Japan). L-NNA was purchased from the Peptide Institute (Osaka, Japan), and L-[2,3,4,5-3H] arginine monohydrochloride (35 to 70 Ci/mmol) was purchased from Amersham (Tokyo, Japan). Tetrahydrobiopterin (BH4) was obtained from Dr. Shircks Laboratories (Joana, Switzerland); Dowex AG 50W-X8 was obtained from BioRad (Richmond, CA), and the BCA protein assay reagent was obtained from Pierce (Rockford, IL). L-NIO was synthesized by us according to the method described by Scannell et al.

Animal Model

The care and maintenance of the rats conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Enotoxin-induced uveitis was induced in 6- to 8-week-old male Lewis rats by footpad injection of 200 μg LPS per rat. According to our previous report, i-NOS activity reached a maximum at 9 hours after LPS injection, and the inhibitors were administered intraperitoneally as doses of 10 mg at 0 hours, 6 hours, or both, after LPS treatment in 0.4 ml physiologic saline solution.

NOS Assay

Nitric oxide synthase activity was measured as described previously. The standard assay mixture (total volume, 50 μl) contained 20 μl of the enzyme solution, 0.1 M Tris buffer (pH 7.5), 1 mM EGTA, 10 mM L-Valine to inhibit arginase, 0.5 μg calmodulin, 1 mM BH4, 1 mM dithiothreitol, 20 mM [3H]-arginine, and 1.25 mM CaCl2. In some experiments, total NOS activity and calcium-independent i-NOS activity were differentially assayed, as previously described, using two different concentrations of CaCl2 to obtain a free-calcium concentration of either 250 μM for total NOS activity or 50 nM for i-NOS activity using calcium–EGTA buffer system. After incubation for 10 minutes at 25°C, assays were terminated with 300 μl of 40 mM Hepes/4 mM EDTA (pH 5.5), and the mixture was applied to 0.4-ml columns of Dowex AG 50W-X8. [3H]citrulline was eluted with 800 μl of distilled water, and the radioactivity was quantitated.

In Vivo Inhibition of Nitric Oxide Synthase in Peritoneal Phagocytes and Aortic Endothelial Cells by L-NNA and L-NIO

Selectivity of the inhibitors was studied in vivo using peritoneal phagocytes and aortic endothelial cells. Because activated phagocytes almost solely express i-NOS activity and unstimulated endothelial cells express a constitutive e-NOS activity, we wanted to confirm the selective effect of the inhibitors of these NOS isozymes after in vivo treatment.

To obtain activated peritoneal phagocytes, 8-week-old Lewis rats were treated with 400 μg LPS by footpad injection, and 10 mg of either L-NNA or L-NIO was intraperitoneally injected 4 hours later. Peritoneal cells were collected aseptically after an additional 3 hours and assayed for i-NOS activity. The cells were...
Aqueous Humor

Aqueous humor samples were collected at times up to 24 hours after LPS injection, at which time the protein concentration in aqueous humor reached maximum. Three series of experiments were designed for each inhibitor; two injections of 10 mg, one at time 0 and one at 6 hours after LPS injection; a single administration of 10 mg of inhibitor at the time of LPS injection or 6 hours later. With the single injections, the dose given at the time of LPS treatment was intended to inhibit NOS in the resident ocular cells, whereas the single injection given at 6 hours after LPS treatment was meant to inhibit i-NOS in activated cells. Aqueous humor was collected from both eyes with a 27-gauge needle under a surgical microscope (approximately 15 μl/rat), and the samples were used for cell count or protein measurement. For the cell count, the AH sample was suspended with an equal amount of 0.4% trypan blue stain solution, and the cells were counted under the microscope. For the protein measurement, each sample was centrifuged to remove the cell pellet, and the protein concentration in the supernatant was measured using BCA protein assay reagent.

Statistical Analysis

Data are expressed as mean ± standard deviation. For statistical analysis, analysis of variance followed by Scheffe F-test was used.

RESULTS

Effects of L-NNA and L-NIO on Phagocytes and Endothelial Cells In Vivo

The effect of 10 mg L-NNA or L-NIO in vivo was studied using NOS assays of activated peritoneal cells and aortic endothelial cells (Fig. 1). In peritoneal cells, L-NNA reduced i-NOS activity to 45% (P < 0.01), whereas L-NIO reduced the activity to 13% (P < 0.005). On the other hand, c-NOS activity in the endothelial cells was reduced by L-NNA to 23% (P < 0.01) and by L-NIO to 54% (P < 0.05). These results show that L-NIO can be used as a relatively selective in vivo inhibitor for i-NOS, whereas L-NNA can act as a relatively selective in vivo inhibitor for endothelial c-NOS (c-eNOS).

Effects of L-NNA and L-NIO on Nitric Oxide Synthase in Ocular Tissue

Figure 2 shows the inhibitory effects of L-NNA and L-NIO on c-NOS and i-NOS activity in ocular tissue. Total NOS activity increases by fourfold to fivefold in EIU, and c-NOS activity apparently also increases in association with total NOS activity. Here, we calculated the c-NOS activity by subtraction of i-NOS activity from total NOS activity. Ocular c-NOS activity was inhibited significantly by both agents, but the inhibition by L-NNA was greater than that by L-NIO (P < 0.01). Ocular i-NOS activity was significantly inhibited only by L-NIO, which reduced the activity to 16%. These results confirm that L-NIO selectively inhibits ocular i-NOS and that L-NNA inhibits ocular c-NOS more effectively than does L-NIO.

Effects of the Inhibitors on Aqueous Humor Protein 24 Hours After Lipopolysaccharide Treatment

The effects of the inhibitors on AH protein are summarized in Figure 3. In EIU, the AH protein increase was 10.5 ± 2.4 mg/ml (mean ± SD, n = 6), whereas two injections of L-NIO at these times gave some inhibition (29%, n = 7), which was not significant. A single injection of L-NNA
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1. Effects of inhibitors on peritoneal phagocyte (A) and aortic endothelial nitric oxide synthase (NOS) activity (B). Asterisks indicate significant differences at \( P < 0.05 \) (*), \( P < 0.01 \) (**), and \( P < 0.005 \) (***) by analysis of variance. (A) Lipopolysaccharide (200 \( \mu g \)) was injected into each footpad, and 10 mg of L-NNA or L-NIO was administered intraperitoneally 4 hours later. Three hours later, the peritoneal cells were collected by washing the peritoneal cavity with phosphate-buffered saline, and NOS activity was measured in washed cells. Data are expressed as mean ± SD (n = 6). (B) A 10-mg dose of L-NNA or L-NIO was injected intraperitoneally, and the aorta was dissected 3 hours later. The tissue was sonicated and, after Sephadex gel filtration, assayed for NOS activity as described in Materials and Methods. Data are expressed as mean ± SD (n = 6).

Effects of the Inhibitors on Cellular Infiltration Into the Anterior Eye

The cell numbers in aqueous humor at 24 hours after LPS injection are shown in Figure 4 (n = 5 for each group). The administration of L-NNA at the time of LPS treatment was as effective as two injections, reducing the protein increase to 4.5 ± 1.8 mg/ml (57% inhibition, \( P < 0.001 \), n = 9), whereas a single injection of L-NNA at 6 hours after LPS treatment had no significant effect (n = 6). A single injection of L-NIO given simultaneously with LPS had no significant effect (n = 14), whereas L-NIO given 6 hours after LPS treatment reduced the protein increase to 7.2 ± 1.8 mg/ml (31% inhibition, \( P < 0.05 \), n = 11), similar to the reduction found with two injections (see above) but which was significant because of the larger n.

Effects of the Inhibitors on Ocular Nitric Oxide Synthase (NOS) Activity

Uveitis was induced as described in Materials and Methods, and the anterior segments of the eyes were collected at 9 hours after lipopolysaccharide treatment. The tissue was sonicated and, after Sephadex gel filtration, assayed differentially for the calcium-dependent (A; c-NOS) and calcium-independent (B; i-NOS) components of total NOS activity as described in Materials and Methods. Data are expressed as mean ± SD (n = 5). Asterisks indicate significant differences at \( P < 0.05 \) (*) and at \( P < 0.01 \) (** by analysis of variance.
FIGURE 3. Effects of inhibitors on aqueous humor protein. Ten-milligram doses of L-NNA or L-NIO were given to endotoxin-induced uveitis rats at 0 and/or 6 hours after lipopolysaccharide (LPS) treatment, and the aqueous humor was collected at 24 hours after LPS treatment to measure the protein content. Data are expressed as mean ± SD (n = 27 for LPS-treated group, n = 6 to 9 for L-NNA treated group, and n = 11 to 14 for L-NIO treated group). Asterisks indicate significant differences at P < 0.05 (*) and at P < 0.001 (**) by analysis of variance.

LPS injection significantly blocked the cellular infiltration with or without a second injection 6 hours later (P < 0.05). The simultaneous administration of L-NIO with LPS decreased the cell number by a variable degree, but the decrease was not statistically significant. The administration of a single dose of either of the inhibitors at 6 hours after LPS injection had no inhibitory effect on cellular infiltration.

Effects of the Inhibitors on Early-Phase Protein Increase
L-NNA and L-NIO were given at the same time as and also at 6 hours after LPS injection, and the aqueous humor was collected at 3 and 9 hours after LPS treatment (n = 3 for each experiment). L-NNA caused a significant inhibition of aqueous humor protein by 3 hours after LPS injection (P < 0.05, Fig. 5).

DISCUSSION
In the current study, we documented the effects of two selective inhibitors of NOS on cellular infiltration and protein extravasation. L-NIO used in our experiments was first tested for its inhibition ability on NOS and was found to be approximately 1 log unit stronger in inhibiting i-NOS in activated phagocyte homogenates than L-NNA (data not shown). Although relative selectivity of L-NNA for c-NOS (e-NOS and neuronal NOS) and L-NIO for i-NOS have been reported,14–17 we first confirmed whether this selectivity applied to NOS inhibition in the rat after in vivo administration (Figs. 1, 2). The endothelial cell–phagocyte NOS inhibition pattern was found to be similar to constitutive–inducible NOS inhibition pattern in ocular tissue for the two inhibitors. Because endothelial cell e-NOS is considered to be the major source of c-NOS in the iris–ciliary body, we speculated that treatment with L-NNA would be more selective than L-NIO for ocular e-NOS. We then selected the time of treatment of these inhibitors based on the previous reports on i-NOS induction. We previously showed that total NOS activity in the iris–ciliary body reaches maximum with the rate of fourfold to fivefold that of an untreated rat 9 hours after LPS treatment,3 and Goureau et al19 reported that expression of i-NOS mRNA is maximum in the iris–ciliary body of the EIU rat at 6 hours after LPS treatment. Thus, inhibitor doses given at time 0 were meant to interact with NOS in the resident ocular cells at the beginning of the disease process, whereas doses given 6 hours later were meant to interact with i-NOS that would be maximally induced in LPS-activated cells at this time.

In our experiments, the most striking differences between the two inhibitors were the degree of cellular infiltration into the anterior chamber (L-NNA almost abrogated cellular infiltration, whereas L-NIO had no significant effect on cellular infiltration) and the effect of simultaneous administration of a single dose of the inhibitors with LPS on cellular infiltration (L-NNA...
FIGURE 5. Effects of nitric oxide synthase inhibitors on early-phase changes in aqueous humor protein levels in endotoxin-induced uveitis rats. The inhibitors were given at 0 and 6 hours after lipopolysaccharide treatment, and the protein content in the aqueous humor was measured at 3 and 9 hours. Data are expressed as mean ± SD (n = 3 for each group). Asterisks indicate significant differences from controls at *P < 0.05, **P < 0.005, and ***P < 0.0005 by analysis of variance.

had a striking effect when given simultaneously with LPS, whereas L-NIO had no significant effect with simultaneous administration). These suggest that the marked effect of simultaneous administration of L-NNA with LPS on cellular infiltration was largely caused by inhibition of e-NOS at a very early stage of the disease course. The inhibition of early-phase AH protein increase, which is clearly shown in Figure 5, is also likely caused by the effect of L-NNA on e-NOS. These results indicate barrier protection by this agent at the beginning stage of the uveitis. By contrast, L-NIO seemed only effective on protein increase when given at 6 hours after LPS treatment (Fig. 5, *P < 0.05) although the same treatment inconsistently seems to increase the cellular infiltration. This may suggest that L-NIO inhibits i-NOS in activated cells regardless of the cellular infiltration process as suggested in a vasculitis model. The increase in cellular infiltration by L-NIO, however, also may indicate that depending on time and dose, inhibitors of NOS could exacerbate a preexisting inflammation, as suggested by others. The effect of L-NIO in inhibiting protein increase was most obvious 9 hours after LPS treatment but was moderate at 24 hours. This suggests that once cellular infiltration occurred, factors other than i-NOS, such as interleukin-6, were involved to increase AH protein at the time of maximal uveitis.

Interestingly, it seems that the degree of ultimate cellular infiltration at 24 hours is determined during the first 6 hours after LPS treatment, because the administration of either inhibitor at 6 hours after LPS treatment had little inhibitory effect on cellular infiltration. This may suggest that NO may be necessary at the very beginning of EIU for the subsequent cellular infiltration process to take place. This finding contradicts the previous reports by Granger and Kubes and Kubes et al that administration of N\textsuperscript{2}-nitro L-arginine methyl ester (L-NAME) induces leukocyte adhesion and that NO is a protective factor for cellular adhesion to endothelium. However, there are other reports in which inhibition of NOS in the inflammatory process leads to inhibition of cellular infiltration; Parks et al reported that inhibition of NOS almost totally abrogated the cellular infiltration in EIU, and Teixeira et al reported that in dermatitis models, the inhibition of NOS blocked cellular infiltration. These latter investigators observed that in the dermatitis models induced by the intradermal injections of bradykinin, zymosan-activated plasma, and other substances, L-NAME inhibits an increase in vascular permeability and cellular infiltration. Furthermore, they observed that L-NIO was less effective than L-NAME in inhibiting dermal edema. The analogy between EIU and dermatitis models is important for understanding the process of barrier breakdown in some types of acute inflammation. Both findings suggest that e-NOS may be involved in the first step of cellular infiltration.

Based on the current study, we can hypothesize that NOS isoforms are involved in the development of EIU in at least two independent sites. One is where e-NOS is involved at the blood–aqueous barrier. Endothelial NOS activity appears to be associated with the increase in protein permeability and seems to promote cellular infiltration. The other is where i-NOS is induced in infiltrating cells and possibly in ocular cells. Infiltrating cells may bring into the eye increased i-NOS activity, together with other inflammatory mediators that promote the inflammation. The inhibition of i-NOS after cellular infiltration is still partially effective, as indicated by the effect of a single injection of L-NIO 6 hours after LPS treatment, but the inhibition of e-NOS in the early phase of EIU is much more important because it abates the cellular infiltration. At present, there is no report on the induction of e-NOS by LPS or nor is there an understanding of how this constitutively expressed enzyme contributes to barrier breakdown. One possibility is that activation of the enzyme increased the interaction of NO with oxygen-free radicals resulting from the inflammation. This can lead to the production of cytotoxic substances, such as peroxynitrite, that cause barrier breakdown. Our study results suggest that activation of e-NOS in barrier cells may play a key role in promoting cellular infiltration in EIU and that inhibition of e-NOS at the early phase of the disease may help minimize the degree of inflammation.
Key Words
endothelin-induced uveitis (EIU), infiltration, N\textsuperscript{c}\textsuperscript{-}nitro L-arginine (L-NNA), N-iminoethyl L-ornithine (L-NIO), nitric oxide synthase

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