Effect of Bacterial Lipopolysaccharide on Ischemic Damage in the Rat Retina

Pablo J. Franco,1 Diego C. Fernandez,1,4 Pablo H. Sande,1 Maria I. Keller Sarmiento,1 Mónica Chbianelli,1 Daniel A. Sáenz,1 and Ruth E. Rosenstein1

PURPOSE. The purpose of this study was to investigate whether bacterial lipopolysaccharide (LPS) induces ischemic preconditioning in the rat retina, and, if so, whether nitric oxide (NO) is involved in this process.

METHODS. Rats were intravitreally injected with different doses of LPS (0.1, 1, or 5 μg) in one eye and vehicle in the contralateral eye 24 hours before retinal ischemia induced by increasing intraocular pressure to 120 mm Hg for 40 or 60 minutes. Subsequently, 7 or 14 days after ischemia, the rats were subjected to electroretinography and histologic analysis. One group of animals received intraperitoneal injections of NOS inhibitors, N-nitro-L-arginine methyl ester (l-NNAME) aminoguanidine or N-(3-aminomethyl)benzyl)acetamidine (W1400) before the injection of LPS or vehicle. Retinal nitric oxide synthase (NOS) activity was assessed through the conversion of 3H-L-arginine to 3H-L-citrulline.

RESULTS. One microgram (but not 0.1 or 5 μg) LPS afforded significant morphologic and functional protection in eyes exposed to ischemia-reperfusion injury. The beneficial effect of LPS was reversed by treatment with l-NNAME, aminoguanidine, or W1400. LPS (1 and 5 μg, but not 0.1 μg) significantly increased retinal NOS activity.

CONCLUSIONS. These results indicate that LPS provides retinal protection against ischemia-reperfusion injury in a dose-dependent manner, probably through an inducible NOS-dependent mechanism. (Invest Ophthalmol Vis Sci. 2008;49:4604–4612) DOI:10.1167/iovs.08-2054

Ischemic preconditioning (IPC) refers to a phenomenon in which brief episodes of ischemia-reperfusion before a prolonged ischemic event limit ischemic damage. IPC has been demonstrated in myocardium,1 brain,2 and liver2 and later in retina.3 Roth et al.1 have shown that IPC affords the retina a greater degree of functional protection against ischemic damage than any known neuroprotective agent, making retinal IPC a particularly attractive area for further research.

Although brief ischemia or hypoxia serve as prototypical IPC stimuli, ischemic tolerance can be induced by exposing animals or cells to a diverse type of endogenous or exogenous stimuli that are not necessarily hypoxic or ischemic, such as hyperbaric oxygenation,5 oxidative stress,6 and hyperthermia,7 among others. Therefore, it seems likely that one stressor can promote cross-tolerance to another. In fact, IPC could also induce protection against nonischemic damage.8 In this context, different preconditioning stimuli such as bright light9 and optic nerve sectioning10 have been shown to protect the retina against light-induced injury.

A small dose of bacterial lipopolysaccharide (LPS) was shown to elicit preconditioning against brain or myocardial ischemic injury11–15 through a process that appears to involve activation of an inflammatory response before ischemia.11–15 However, so far, the hypothesis that LPS may induce tolerance against retinal ischemia-reperfusion injury has not been investigated.

Some requirements for the induction and/or expression of retinal IPC have been established16–18 but the mechanisms involved in this response remain incompletely understood. It has been shown that IPC can be produced by pathophysiological stressors, including adenosine,16 ATP-sensitive potassium channels (KATP),16–18 opioids,19 and free radicals,6,20 among many other signals (for a review, see Ref. 21). Moreover, several studies have demonstrated that retinal IPC involves the generation of nitric oxide (NO).22,23 It is well known that LPS drives inducible (i)NOS gene expression in various systems, including the retina,24 leading to an increase in NO levels. Therefore, in the present study, we hypothesized that LPS may trigger IPC in the retina and that iNOS is involved in this process.

MATERIAL AND METHODS

Animals

Male Wistar rats (average weight, 250 ± 40 g) were housed in a standard animal room with food and water ad libitum in controlled conditions of humidity and temperature (21 ± 2°C), with a 12-hour light–12-hour dark lighting schedule (lights on at 7 AM). These studies were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. A total of 222 animals were used for the experiments as follows: 30 rats for assessment of protein and cell count in the aqueous humor; 42 for ERG assessment and retinal histology (effect of LPS per se); 40 for the effect of LPS on ischemia–reperfusion damage; 60 for the effect of l-NNAME, aminoguanidine, and W1400; and 50 for NO activity studies. In addition, a group of 20 animals anesthetized and cannulated without raising intraocular pressure (IOP) were used in control experiments.

LPS Preconditioning Protocol

Animals were anesthetized with ketamine hydrochloride (150 mg/kg) and xylazine hydrochloride (2 mg/kg) administered intraperitoneally. A drop of proparacaine (0.5%) was administered in each eye for local anesthesia. With a syringe (Hamilton, Reno, NV) and a 30-gauge needle, 2 μL of sterile, pyrogen-free saline containing 0.1, 1, or 5 μg LPS...
from Salmonella typhimurium (catalog no. L-7261; Sigma-Aldrich, St. Louis, MO) were injected into one eye of anesthetized rats, and an equal volume of vehicle was injected into the fellow eye. Injections were applied at 1 mm of the limbus and the needle was left in the eye for 60 seconds; this small volume prevented an increase in IOP and volume loss.

**Integrity of the Blood–Ocular Barrier**

Animals were euthanatized, and the aqueous humor was collected immediately from each eye by anterior chamber puncture with a 30-gauge needle under a surgical microscope. Cell count and protein concentration in aqueous humor samples obtained 24 hours or 7 days after the injection of vehicle or LPS were assayed to assess blood–ocular barrier integrity. The number of cells was manually counted with a Neubauer hemocytometer, and the number of cells per micro-liter was obtained by averaging the results of four fields from each sample. Protein concentration in aqueous humor was determined by the method of Lowry et al., using bovine serum albumin as the standard. Aqueous humor samples were stored on ice until testing, and cell counts and protein concentrations were measured on the day of sample collection.

**Ischemia Methodology**

One day after intravitreous injections of vehicle or LPS, pupils were dilated in anesthetized rats. After topical instillation of proparacaine, the anterior chamber of both eyes was cannulated with a 30-gauge needle connected to a bottle filled with saline solution. Retinal ischemia was induced by elevating the saline reservoir above the eye, thereby increasing IOP to 120 mm Hg. The increased IOP was maintained for exactly 40 or 60 minutes. With this maneuver, complete ocular ischemia was produced, characterized by loss of the ERG b-wave and cessation of flow in retinal vessels, determined by fundoscopic examination. During and after (before returning the rats to the animal house) the experiments, animals were kept normothermic with heated blankets. All rats were allowed at least 7 days of reperfusion after sustained ischemia. A few animals in which cataracts developed due to lens injury, were not used any further in the experiments. In the present study, vehicle-injected eyes served as the control group, because in preliminary studies we found that in comparison with intact animals, the injection of vehicle did not affect the ERG (without ischemia) or the morphologic and functional retinal damage induced by ischemia-reperfusion (data not shown).

**Ischemic Preconditioning Inhibitors**

To assess the involvement of NOS in the ischemic protection afforded by LPS, rats received vehicle (saline solution), N-nitro-L-arginine methyl ester (L-NAME, 50 mg/kg), aminoguanidine (100 mg/kg), or N-(3-aminomethyl)benzyl)acetamidine (W1400, 10 mg/kg). L-NAME and W1400 were administered intraperitoneally in a single dose 30 minutes before LPS, and aminoguanidine was administered 30 minutes before and 6 hours after LPS. The timing and dose of aminoguanidine administration was selected on the basis of a report by Kawano et al., whereas the dose of W1400 was chosen according to Kobata et al.

**Electroretinography**

ERG activity was assessed before ischemia and 7 or 14 days after ischemia, as follows: After 6 hours of dark adaptation, the rats were anesthetized under dim red illumination. Phenylephrine hydrochloride and tropicamide were used to dilate the pupils, and the cornea was intermittently irrigated with physiologic saline to maintain the baseline recording and to prevent keratopathy. Rats were placed facing the stimulus at a distance of 20 cm. All recordings were completed within 20 minutes, and the animals were kept warm during and after the procedure. A reference electrode was placed through the car, a grounding electrode was attached to the tail, and a gold electrode was placed in contact with the central cornea. A 15-watt red light was used to enable accurate electrode placement. This maneuver did not significantly affect dark adaptation and was switched off during the electro-physiological recordings. ERGs were recorded from both eyes simultaneously, and 10 responses to flashes of unattenuated white light (5 ms, 0.2 Hz) from a photostimulator (light-emitting diodes) set at maximum brightness (350 cd/s/m² without filter) were amplified, filtered (1.5-Hz low-pass filter, 1000-Hz high-pass filter, notch-activated), and averaged (Bio-PAC; Akonic, Buenos Aires, Argentina). The a-wave was measured as the difference in amplitude between the recording at onset and the trough of the negative deflection, and the b-wave amplitude was measured from the trough of the a-wave to the peak of the b-wave. Electrophysiological responses were averaged for each run. Runs were repeated three times with 5-minute intervals, to confirm consistency, and the mean of these three runs was used for subsequent analysis. The mean peak latencies and peak-to-peak amplitudes of the responses from each group of rats were compared. Baseline (preischemic) recordings were taken at least 1 day before treatment.

**Histologic Evaluation**

Fourteen days after ischemia, the rats were killed, and their eyes were immediately enucleated, immersed for 24 hours in a fixative containing 4% formaldehyde in 0.1 M phosphate buffer (pH 7.2), and embedded in paraffin. The eyes were sectioned (5 μm) along the vertical meridian through the optic nerve head. Microscopic images were digitally captured (Eclipse E400 microscope with illumination; 6-V halogen lamp, 20 watts, equipped with a stabilized light source; Nikon, Tokyo, Japan, via a Coolpix s10 camera; Nikon). The sections were stained with hematoxylin and eosin and analyzed by masked observers. The average thickness (in micrometers) of the retina and retinal layers for each eye was measured. The number of cells in the ganglion cell layer (GCL) was calculated by linear cell density (cells per 200 μm). No attempt was made to distinguish cell types in the GCL for enumeration of the cells. Measurements (×400) were obtained at 1 mm dorsal and ventral of the optic disc. For each eye, results obtained from four separate sections were averaged, and the mean of five eyes was recorded as the representative value for each group. For histologic studies of the effect of LPS per se on anterior segment and retina, animals were killed 24 hours or 7 days after injection of vehicle or LPS, the eyes were routinely processed with paraffin, and sections were stained with hematoxylin and eosin.

**NOS Activity Assessment**

Retinal NOS activity was assessed as previously described in retinas from eyes injected with vehicle or LPS, in the presence or absence of calcium. One day after injections, each retina was homogenized in 100 μL of buffer solution containing 0.32 M sucrose and 0.1 mM EDTA (adjusted to pH 7.4 with Tris base). Reaction mixtures contained 50 μL of the enzyme source and 50 μL of a buffer stock solution (final concentrations: 10 mM HEPES, 1 mM NADPH, 5 μM FAD, 1 mM β-mercaptoethanol, and 25 mM L-valine, an arginase inhibitor), 10 μM L-arginine (5 μCi/mL), and 1 μM t-arginine, with or without 3 mM CaCl₂. In the absence of calcium, 5 mM EGTA was added to the reaction mixture. After incubation at 37°C for 30 minutes, the reaction was stopped by adding 200 μL of stop buffer (50 mM HEPES, 10 mM EDTA, and 10 mM EGTA; pH 5.5), and the tubes were cooled for 5 minutes. The solution was mixed with 600 μL of Na⁺ resin (Dowex AG50W-X8; Bio-Rad Laboratories, Hercules, CA) to remove t-arginine, and centrifuged at 10,000 g for 5 minutes. The resins were added to the supernatant and were quantified by liquid scintillation counting. Nonenzymatic conversion of 3H-L-arginine to 3H-citrulline was tested by adding buffer instead of the enzyme source.

Statistical analysis of the results was made by Student’s t-test or a two-way analysis of variance (ANOVA) followed by the Tukey test, as stated. Parametric tests were used throughout, since data met the requirements for those tests (no significant differences in standard
deviations among groups, and data populations that followed a Gaussian distribution).

RESULTS

Figure 1 depicts protein concentration (Fig. 1A) and infiltrated cell count (Fig. 1B) in the aqueous humor from eyes intravitreally injected with vehicle in one eye and LPS (0.1, 1, or 5 μg) in the contralateral eye. After 24 hours of injection, 1 and 5 μg LPS significantly increased both parameters. However, the increase in protein levels and infiltrated cell number was significantly higher \((P < 0.01)\) with 5 μg than with 1 μg LPS, whereas 0.1 μg LPS did not show any significant effect. No changes in aqueous humor protein concentration and infiltrated cell count were observed 7 days after the intravitreous injection of any dose of LPS (data not shown). The effect of LPS injection per se on the scotopic ERG and anterior segment and retinal histology were assessed both at 24 hours and 7 days after injections. After 24 hours of injection, no significant changes in the ERG were found for all the examined doses of LPS, as shown in the top panel of Figure 2. Similar results were observed 7 days after LPS injection (data not shown). After 24 hours, the injection of 5 μg LPS induced severe inflammation signs affecting the iris-ciliary body, limbus, and vitreous humor, showing massive inflammatory cell infiltration (Fig. 2C). In eyes injected with 1 μg LPS (Fig. 2B), the inflammatory signs in the anterior chamber and vitreous humor were milder than those observed with 5 μg LPS. No significant signs of inflammation were observed at the retinal level in eyes injected with 1 or 5 μg LPS (Figs. 2E, 2F, respectively). The injection of vehicle (Figs. 2A, 2D) and the dose of 0.1 μg LPS (data not shown) did not provoke significant inflammation in the anterior segment or retina. After 7 days of 0.1, 1, or 5 μg LPS injection, no significant inflammation was observed in the anterior chamber or retina (data not shown).

To analyze the effect of LPS on ischemia-reperfusion injury, vehicle or LPS (0.1, 1, or 5 μg) were injected 24 hours before ischemia induced by increasing IOP to 120 mm Hg for 40 minutes, and the ERG was assessed 7 days after ischemia. Representative scotopic ERG traces from rats injected with vehicle or 1 μg LPS and 24 hours after injections exposed to 60 minutes of ischemia are shown in Figure 5A. The average amplitudes of scotopic ERG a- and b-waves before ischemia or 7 days after 40-minute ischemia in eyes injected with vehicle in one eye and LPS in the contralateral eye 24 hours before ischemia are depicted in the same figure. In eyes injected with vehicle, ischemia for 40 minutes and reperfusion for 7 days induced a significant \((P < 0.01)\) decrease in ERG a- and b-wave amplitudes, whereas their latencies remained unchanged. The ischemia-induced decrease in both a- and b-wave amplitudes was significantly reduced in eyes which received an intravitreous injection of 1 μg LPS. Compared with eyes injected with vehicle, a further decrease in ERG a- and b-wave amplitudes was observed in eyes receiving 5 μg LPS, whereas 0.1 μg LPS did not show any significant effect. Similar results were observed when the ERG was assessed 14 days after ischemia (data not shown). No significant differences were observed between noninjected and vehicle-injected eyes.

Figure 4 shows photomicrographs of the typical rat retina. Figure 4A shows a representative photomicrograph of the nonischemic control group. Fourteen days after 40 minutes of ischemia, typical pathologic changes were seen in the retina from eyes injected with vehicle 24 hours before ischemia (Fig. 4B). Marked reductions in the thickness of the inner plexiform layer (IPL), outer nuclear layer (ONL), inner and outer segments of photoreceptors (PR), and cell density in GCL were observed in eyes injected with vehicle (Fig. 4B), 0.1 (Fig. 4C), or 5 μg LPS (Fig. 4E) and exposed to 40 minutes of ischemia 24 hours after injections. In contrast, 1 μg LPS significantly inhibited this reduction in the thickness of IPL, ONL, and PR and in GCL cell density (Fig. 4D, Table 1).

The protective effect of 1 μg LPS against ischemia-reperfusion injury was also evident when ischemia was induced by increasing IOP up to 120 mm Hg for 60 minutes. Representative scotopic ERG traces from rats injected with vehicle or 1 μg LPS and 24 hours after injections exposed to 60 minutes of ischemia are shown in Figure 5A. The average amplitudes of scotopic ERG a- and b-waves before ischemia or 7 days after 60 minutes of ischemia in eyes injected with vehicle in one eye and 1 μg LPS in the contralateral eye 24 hours before ischemia are depicted in Figure 5B. Ischemia for 60 minutes and reperfusion for 7 days induced a further decrease in both ERG a- and b-wave amplitudes compared with damage induced by the 40-minute ischemia \((P < 0.01)\). The intravitreal injection of 1 μg LPS 24 hours before the 60-minute ischemia significantly reduced the ERG dysfunction. The protective effect of 1 μg LPS was evident also at the histologic level, as shown in the images at the bottom of the same figure. In comparison with the nonischemic retina (Fig. 5C), retinas subjected to 60 minutes of ischemia 24 hours after injection of vehicle showed profound structural alterations. The thicknesses of the total retina were: 34.5 ± 4.3 and 99.7 ± 3.6 μm for vehicle and 1 μg LPS, respectively \((P < 0.01)\). A widespread retinal disorganization rendered it impossible to discern boundaries between layers for accurate measurement of layer thickness or quantitative cell counts. Retinas treated with 1 μg LPS 24 hours before the
60-minute ischemia (Fig. 5E) demonstrated structural protection in comparison to vehicle-treated eyes.

To analyze the involvement of NOS activity in ischemic tolerance induced by LPS, we injected the animals with NOS inhibitors, L-NAME, aminoguanidine, or W1400 and 24 hours after injection of LPS, the eyes were exposed to 40 minutes of ischemia. As shown in Figure 6, L-NAME, aminoguanidine, and W1400 significantly abrogated the ischemic preconditioning induced by LPS. L-NAME, aminoguanidine, and W1400 per se did not induce any significant ERG changes in retinas damaged by ischemia-reperfusion (data not shown).

The effect of LPS (0.1, 1, or 5 μg) on retinal NOS activity in the presence or absence of Ca²⁺ is shown in Figure 7. Twenty-four hours after injection, LPS induced a dose-dependent increase in retinal NOS activity in both experimental conditions. In the presence or absence of calcium, the lowest dose did not affect the retinal biosynthesis of NO, whereas 1 and 5 μg increased this parameter, with 5 μg LPS being significantly more effective than 1 μg. The absence of calcium significantly decreased NOS activity in all experimental groups.

**DISCUSSION**

For the first time, we have conducted experiments showing that LPS protects both retinal function and histology from ischemia-reperfusion injury in a dose-dependent manner, most likely through an iNOS-dependent mechanism.

In the present report we increased IOP to induce retinal ischemia. In contrast to the vascular ligation model, this strategy, which has been used in other retinal IPC experiments, has the advantage that no surgical procedure is needed to induce retinal ischemia. After IOP was increased to 120 mm Hg for 40 minutes, a significant impairment of retinal function (revealed by ERG) was observed in vehicle-injected eyes. Compared to preischemic amplitudes, the a- and b-wave amplitudes were strongly reduced 7 days after 40 minutes of ischemia (by ~50%, and ~40%, for a- and b-wave, respectively, P < 0.01). Moreover, ischemia-reperfusion induced significant histologic alterations. In agreement with Ettaiche et al., the retinal dysfunction was time-of-hypertension-dependent, since a greater retinal injury (P < 0.01) was provoked by the 60-minute ischemia (~10% and ~7% of baseline a- and b-wave amplitudes, respectively), than by the 40-minute ischemia. In agreement, the histologic injury was greater with the 60-minute than with the 40-minute ischemia.

The intravenous, intraperitoneal, intravitreous, or footpad administration of LPS induced an acute inflammatory response in the uveal tract, generally termed endotoxin-induced uveitis (EIU), which is characterized by an early breakdown of the blood-aqueous barrier and the subsequent development of clinical signs and cellular infiltrate. The reaction peaked at 24 hours.
hours after injection (for a review, see Refs. 32, 33). Vascular permeability and clinical and pathologic changes induced by LPS have been reported to be dose dependent. In agreement, the intravitreous injection of 1 and 5 μg LPS induced a significant increase in aqueous humor protein content and number of cells (an index of blood–aqueous barrier disruption), but 5 μg LPS was significantly more effective than 1 μg, whereas 0.1 μg LPS showed no effect. At 24 hours or 7 days after injection, no significant changes in ERG activity were induced by 0.1, 1, or 5 μg LPS. In agreement, after injections of 1 or 5 μg LPS, no significant signs of inflammation were observed at the retinal level, whereas a pronounced inflammation was evident in the eye anterior segment, as previously reported.

Although 1 and 5 μg LPS provoked a significant disruption of the blood–aqueous barrier, only 1 μg LPS elicited significant retinal protection against ischemia–reperfusion injury at both the functional and histologic levels, whereas 5 μg LPS provoked a further decrease in ERG a- and b-wave amplitudes and an apparently greater retinal disorganization. Taking into account the lack of effect of 5 μg LPS per se on the ERG, the reasons for the worsening in retinal function and histology induced by this dose of LPS are not clear. However, it seems possible that 5 μg LPS provoked subclinical retinal alterations that could be potentiated by ischemia–reperfusion.

It has been postulated that differences in the intensity, duration, and/or frequency of a particular stress stimulus determine whether that stimulus is too weak to elicit a response, sufficient in magnitude to serve as a preconditioning trigger, or too robust and therefore harmful. The present results seem to fit into this paradigm, with the lowest dose of LPS (0.1 μg) too weak, the medium dose (1 μg) effective as a preconditioning stimulus, and the highest dose (5 μg) injurious. In agreement with these results, it has been shown that LPS pretreatment induces myocardial and cerebral protection against ischemia–reperfusion injury, and it has been used as a preconditioning stimulus in a mouse model of middle cerebral artery occlusion.

In vivo experiments have shown that IPC provides functional (attenuation of ERG changes) and histologic protection after prolonged retinal ischemia, although reports differ considerably in the degree of functional protection afforded. As shown herein, the beneficial effect of 1 μg of LPS was highly significant, reaching a ~98% and ~80% of protection for a- and b-waves.
Ischemic Tolerance Induced by LPS

Preconditioning in the rat retina. In contrast, Zhu et al.22 involved in the histologic protection induced by ischemic preconditioning, suggesting that NO synthesized by iNOS is a protective mediator of IPC, in several tissues, including the retina. Pharmacologic and genetic evidence supporting the involvement of NO (derived from the endothelial, 39,40 neuronal, and inducible12 isoforms of NOS) in the transcription of IPC has been provided. Sakamoto et al.25 have shown that treatment of rats with aminoguanidine and L-N6-(1-iminoethyl) lysine, but not N\(^{-\text{G}}\) nitro-L-arginine, N\(^{-\text{G}}\) monomethyl-L-arginine, or 7-nitroindazole, abolished the protective effect of IPC, suggesting that NO synthesized by iNOS is involved in the histologic protection induced by ischemic preconditioning in the rat retina. In contrast, Zhu et al.22 showed that ischemic preconditioning was completely effective in iNOS knockout mice, whereas ischemic tolerance was not achieved in retinas from endothelial (e)NOS and neuronal (n)NOS knockout mice. As shown herein, the beneficial effects of LPS were blocked by a nonselective NOS inhibitor (L-NAME), as well as by aminoguanidine and W1400, which are selective inhibitors of iNOS. The abrogation of protection against retinal ischemic damage cannot be attributed to an intrinsic deleterious effect of L-NAME, aminoguanidine, or W1400 pretreatment, because administration of these compounds had no influence on the ERG changes induced by ischemia-reperfusion.

It was demonstrated that intravenous administration of LPS induces iNOS in the retina at both the RNA and protein levels.45,46 The present results support these observations, since LPS significantly increased calcium-independent retinal NOS activity. Moreover, it has been reported that LPS triggers protection against myocardial infarction77 and in the mouse, against cerebral artery occlusion,78 via iNOS. Thus, without excluding the activation of other transduction pathways described in retinal IPC by other groups such as adenosine,18,48,49 K\(_{\text{ATP}}\) channels,18,19,48,49 PKC,48,49 and vascular endothelial growth factor,50 among others, these results support that iNOS-derived NO could represent a crucial step in retinal preconditioning induced by LPS. Of interest, it has been postulated that NO derived from iNOS could be an upstream regulator of the mitochondrial K\(_{\text{ATP}}\) channel in cardioprotection by preconditioning.51

Although in several reports, the involvement of NO/NOS in IPC was supported by immunohistochemical, genetic, or pharmacologic strategies, a necessary (but not sufficient) condition to corroborate the participation of NO is to demonstrate an upregulation of NO biosynthesis in the paradigm of retinal ischemic tolerance, which was not previously provided. The present results indicated that 1 and 5 \(\mu\)g LPS (but not 0.1 \(\mu\)g LPS) significantly increased retinal NOS activity, but the dose of 5 \(\mu\)g LPS was significantly more effective than 1 \(\mu\)g, both in the presence and absence of calcium. However, as already mentioned, only 1 \(\mu\)g LPS was effective as an IPC stimulus. Based on these results and on other reports (for a review, see Ref. 52), it is tempting to speculate that NO may have a dual role, being protective or destructive, depending on its concentration. In this way, by increasing NO biosynthesis, 1 \(\mu\)g LPS could induce retinal protection, whereas by further increasing NO levels, 5 \(\mu\)g LPS could potentiate retinal damage.

Because NOS activity was assessed in the whole retina, we could not ascertain the locus of the observed phenomenon. In addition, the possibility that the infiltrating cells are the source of NO cannot be ruled out. However, NO diffuses fairly freely across cell membranes, and it could reach different cells, beyond those in which it is produced. The fact that LPS-induced protection was observed on both ERG a- and b-waves and on

**TABLE 1.** Retinal and Retinal Layer Thickness from Eyes Injected with Vehicle or 1 \(\mu\)g LPS 24 Hours before 40 Minutes of Ischemia and Assessed 14 Days after Ischemia

| Thicknesses (\(\mu\)m, mean \(\pm\) SEM, \(n = 5\) retinas/group) of total, the whole retina; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; OPL, outer nuclear layer; PR, inner and outer segment of photoreceptors, and cell count (mean \(\pm\) SEM) in ganglion cell layer (GCL). |
|-----------------|-----------------|-----------------|-----------------|
| Nonischemic Group | Vehicle + 40-min Ischemia | 1 \(\mu\)g LPS + 40-min Ischemia |
| Total | 153.8 \(\pm\) 3.7 | 75.5 \(\pm\) 4.2** | 115.6 \(\pm\) 5.0** |
| IPL | 51.0 \(\pm\) 1.4 | 16.5 \(\pm\) 2.3** | 22.4 \(\pm\) 3.7 |
| INL | 17.3 \(\pm\) 1.6 | 9.2 \(\pm\) 2.8** | 16.8 \(\pm\) 1.0** |
| OPL | 8.0 \(\pm\) 1.0 | 5.5 \(\pm\) 0.6 | 6.4 \(\pm\) 0.5 |
| ONL | 29.6 \(\pm\) 1.1 | 19.8 \(\pm\) 0.9** | 30.2 \(\pm\) 0.5** |
| PR | 38.8 \(\pm\) 4.2 | 15.3 \(\pm\) 1.1** | 26.8 \(\pm\) 1.8** |
| GCL | 14.6 \(\pm\) 0.6 | 7.0 \(\pm\) 0.5** | 13.4 \(\pm\) 1.0** |

**P < 0.05 and **P < 0.01 versus nonischemic group.

**P < 0.05 and **P < 0.01 versus vehicle-injected eyes, by the Tukey test.
most of the retinal layers, suggests that this protection could be a panretinal phenomenon. One difference observed between previous results from other groups and our findings resides in the ONL thickness. Using a similar model of retinal ischemia, Sakamoto et al. did not find changes in the ONL thickness after ischemia, whereas Roth et al. (using the vascular ligation model) showed a mild disorganization of this layer. In contrast to these reports, we showed a significant reduction of this parameter 14 days after 40 minutes of ischemia that was significantly abrogated by 1 µg LPS injected 24 hours before ischemia. Those reports differ from our work in two principal respects: (1) The rat strains were different (Wistar rats in our work and Sprague-Dawley in the other studies); and (2) whereas in those studies, the histologic analysis was performed 7 days after ischemia, our experiments were performed 14 days after ischemia. A different sensitivity to retinal ischemia has been observed in the different rat strains. On the other hand, although several studies of the postischemic retina have reported that cell death is most pronounced in the inner retina, results of other studies indicate that with extended ischemia
Figure 7. The effect of intravitreal injection of LPS on retinal NOS activity assessed in the presence or absence of calcium. Retinal conversion of L-arginine to L-citrulline was assessed 24 hours after injection of vehicle or LPS (0.1, 1, or 5 μg). A significant increase of this parameter was observed in retinas of eyes injected with 1 or 5 μg LPS (both in the absence and presence of calcium), but not in those injected with 0.1 μg LPS. NOS activity was significantly higher with 5 μg LPS than with 1 μg in both experimental conditions. The absence of calcium significantly decreased retinal NOS in all experimental groups (P < 0.01, Student’s t-test). Data are the mean ± SEM (n = 18–20 animals/group in the presence of calcium, n = 10 animals/group in the absence of calcium). *P < 0.01 versus NOS activity from vehicle-injected eyes assessed with the same concentration of calcium; **P < 0.01 and ***P < 0.05 versus 1 μg LPS, by the Tukey t-test.

postischemic survival times, cell death becomes significant in the ONL. 55–57 Thus, both differences could account for the discrepancy between the present results and those in the aforementioned studies.

Severe visual disability or blindness may follow retinal ischemia. Although investigators have performed studies to examine the ability of exogenous agents to prevent damage after retinal ischemia, so far, none of these strategies has been completely effective. The potential use of the retina’s endogenous system to protect against ischemia deserves to be examined to find new therapeutic strategies for ischemic retinal diseases. In this context, the demonstration that a moderate retinal inflammation induces ischemic tolerance could constitute a future fertile avenue for the development of new therapeutic strategies in the treatment of retinal ischemic damage.

References