Effects of Rolipram, a Selective Inhibitor of Type 4 Phosphodiesterase, on Lipopolysaccharide-Induced Uveitis in Rats

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PURPOSE. To investigate effects of rolipram, an inhibitor of type 4 phosphodiesterase, on lipopolysaccharide (LPS)-induced uveitis in Wistar rats.

METHODS. A total of 100 μg LPS was injected into the rat footpad. Rolipram (Wako Pure Chemical, Osaka, Japan) was injected intraperitoneally 30 minutes before administration of LPS. Levels of intracamerat protein, cells, E-selectin, tumor necrosis factor (TNF)-α, interleukin (IL)-6, and nitrite were determined. E-selectin, TNF-α, IL-6, and inducible nitric oxide synthase (iNOS) mRNAs and immunohistochemical reactivity of nuclear factor (NF)-κB and TNF-α were also examined in the iris-ciliary body.

RESULTS. After LPS injection, intracameral protein and cells increased from 18 to 30 hours later. Rolipram, however, inhibited elevation of protein and cells. After LPS injection, mRNA levels of E-selectin, TNF-α, and IL-6 in the iris-ciliary body increased 3 hours later, and iNOS mRNA increased 6 hours later. Elevation of mRNA levels for E-selectin, TNF-α, and IL-6 was inhibited by rolipram. After LPS injection, intracameral TNF-α and IL-6 levels increased 4 to 6 hours later, and nitrite levels increased 14 to 20 hours later. Elevation of TNF-α and IL-6 levels was decreased by rolipram. Rolipram did not affect iNOS mRNA and nitrite levels. Immunoreactivity of NF-κB was strong 4 hours after LPS injection and was decreased by rolipram.

CONCLUSIONS. NF-κB translocation and expression of E-selectin, TNF-α, and IL-6 are involved in the pathogenesis of LPS-induced uveitis and are inhibited by rolipram. The inhibitory effect of rolipram in uveitis may be independent of iNOS synthesis. (Invest Ophthalmol Vis Sci. 2004;45:2497–2502) DOI:10.1167/iovs.03-1373

Endotoxin (lipopolysaccharide; LPS)-induced uveitis in rats is a known model of human disease. Interleukin (IL)-6, tumor necrosis factor (TNF)-α, E-selectin, and nitric oxide (NO) production have been reported to be involved in the pathogenesis of LPS-induced inflammation and uveitis. IL-6 and TNF-α are known as proinflammatory cytokines, and E-selectin contributes to continuing cellular infiltration into the site during inflammation. Immunologic and inflammatory stimuli induce the expression of the inducible isoform of nitric oxide synthase (iNOS), producing NO. LPS has induced translocation of nuclear factor (NF)-κB, an inducible transcription factor that mediates the overproduction of TNF-α and other cytokines.

Phosphodiesterase type 4 is reportedly a major cAMP-hydrolyzing isoenzyme in proinflammatory cells, including T-lymphocytes, monocytes, neutrophils, and eosinophils. Rolipram, a type 4 phosphodiesterase inhibitor, suppresses cutaneous inflammation, zymosan-induced inflammation, LPS-induced TNF-α expression, and collagen-induced arthritis. Xu et al. have demonstrated the protective effect of rolipram in experimental autoimmune uveoretinitis and have reported that the protection is independent of IL-10–induced activity. Blease et al. have reported that rolipram, in combination with salbutamol inhibited TNF-α, induces E-selectin expression. Sanz et al. have demonstrated that rolipram inhibits leukocyte-endothelial cell interactions in vivo through P- and E-selectin downregulation. Rolipram is used as an antidepressant.

In the present study, in a model of LPS-induced uveitis in Wistar rats, we determined the number of cells and amount of protein in the aqueous humor and the mRNA and protein levels of IL-6, TNF-α, E-selectin, and nitrite and of NF-κB.

MATERIALS AND METHODS

Animals

A total of 206 male Wistar rats (6–8 weeks old, 170–200 g) was obtained from Sankyo Labo Service Co., Inc. (Tokyo, Japan). The animals were housed in 12-hour light–dark conditions and were given food and water ad libitum during the experiment. All studies were conducted in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The study was approved by the Institutional Committee for the Ethics in Animal Care and Treatment.

Injection of LPS and Rolipram

To produce uveitis, LPS (Escherichia coli, serotype 055:B5; Sigma-Aldrich, St. Louis, MO) in 100 μg/100 μL pyrogen-free 0.9% sodium chloride was injected into one hind footpad. Rats treated with 0.9% sodium chloride alone served as the control. Rolipram (4-(3-cyclopentyl-4-methoxyphenyl)-2-pyrrolidinone) was purchased from Wako Pure Chemical (Osaka, Japan), dissolved in 5% dimethyl sulfoxide (DMSO), and injected intraperitoneally 30 minutes before administration of LPS. The treatments were performed between 9 and 11 AM.

Measurement of Protein and Number of Cells in the Aqueous Humor

Aqueous humor was aspirated several hours after injection of LPS. After intraperitoneal injection of pentobarbital (50 mg/kg body weight) aqueous humor was aspirated with a 30-gauge needle under visualization with a microscope. Aspirated samples were centrifuged at 1500 rpm for 5 minutes at 4°C to obtain the supernatant. Protein
concentration in the supernatant was measured by a protein-dye-binding assay, as described by Bradford, and was expressed relative to a bovine serum albumin standard. To 5 μL of aspirated aqueous humor the same volume of 0.4% trypan blue was added. The stained cells were then counted with a hemocytometer.

RNA Extraction and Real-Time Polymerase Chain Reaction

After intraperitoneal injection of pentobarbital (50 mg/kg body weight) the eye globe was enucleated and immediately submerged in RNA stabilization reagent (RNALater; Qiagen, Hilden, Germany), followed by isolation of the iris-ciliary body tissue from the stabilized eye globe. The dissected iris-ciliary body tissue was homogenized with a rotor-stator homogenizer in buffer (RLT; Qiagen). Total RNA was extracted with a kit (RNeasy Protect Mini Kit; Qiagen) and treated with a reverse transcriptase with RNAse-free DNase (Qiagen) to remove any residual genomic DNA. cDNA from each sample was obtained by reverse transcription with random hexamers using reverse transcriptase (Transcriptor; Applied Biosystems Inc., [ABI], Tokyo, Japan).

Based on the database, real-time PCR primers and probes (Nippon EGT, Tokyo, Japan) were designed for E-selectin (GenBank accession no. L25527; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD; forward primer 5′-GCCATGTTGCTGTAATGTAAAGG-3′, reverse primer 5′-GGATTGGAGACATTGCTGACT-3′, and 5′-FAM)TGACCCAACCTGGCAGTGACCTGA(TAMRA)-3′ (TaqMan probe; ABI); for TNF-α (accession no. NM012675): forward primer 5′-ACAAAGGCTGCAGCCGACTACG-3′, reverse primer 5′-TCTCTGTTATGAAATGGCAAAC-3′, and probe 5′-FAM)TGCTCTCAACCAACGCGTCAGCTG(C(TAMRA))-3′ (TaqMan; ABI); for IL-6 (accession no. NM012589): forward primer 5′-TCAACTCTCATCTGCCCTTCAG-3′, reverse primer 5′-AAGGCACCTGGCTGAAGGTCT-3′, and 5′-FAM)AAACAGCTATGAGTTCTGCTCCGACAT(TAMRA)-3′ (TaqMan probe; ABI); for iNOS (accession no. NM012611): forward primer 5′-TGGTCCACCTGAGGTCTTT-3′, reverse primer 5′-CAGTAATGGCCGACCTGGATGT-3′, and 5′-FAM)TGCCGCGAGCTGGTACCTGAT(TAMRA)-3′ (TaqMan probe; ABI); and for glyceraldehyde 3-phosphate dehydrogenase (GAPDH, accession no. AF106860): forward primer 5′-GATGTTCAAACGACCTGGATGT-3′, reverse primer 5′-GCTTTGAAGGCTACCTGACCTGAT-3′, and 5′-FAM)CATCTGGGCTACACTGAGGACCA(TAMRA)-3′ (TaqMan probe; ABI). cDNA was used to detect real-time PCR products for E-selectin, TNF-α, IL-6, and iNOS with master mix (TaqMan Universal Master Mix; ABI) and a sequence detection system (Prism 7700; ABI) with specific primers and probe. The thermal profile for each primer consisted of 2 minutes at 50°C and 10 minutes at 95°C, followed by 40 cycles for 15 seconds at 95°C and 1 minute at 60°C. To compare expression patterns, mRNA template concentrations of GAPDH and the target genes were calculated using the standard curve method. The expression levels of E-selectin, TNF-α, IL-6, and iNOS mRNA were normalized by the GAPDH mRNA level in each sample, and the changes were expressed as an n-fold increase, relative to the levels in control rats treated with 0.9% NaCl alone.

Protein Levels of TNF-α and IL-6 in the Aqueous Humor

After LPS injection, aqueous humor was aspirated with a 30-gauge needle under microscopic visualization. Aspirated samples were centrifuged at 2500 rpm for 20 minutes at 4°C to obtain the supernatant. Protein levels of TNF-α and IL-6 were determined by an enzyme-linked immunosorbent assay (AN’ALYZA; Techne Corp., Minneapolis, MN).

Nitrite Levels in Aqueous Humor

The nitrite level in the aqueous humor was measured colorimetrically by using a nitrite oxide assay kit (EMD Biosciences, Darmstadt, Germany), according to the manufacturer’s instruction. The aqueous humor from both eyes of a rat was diluted up to 40 μL and used for one assay.

Immunohistochemistry for Subunit p65 of NF-κB and TNF-α

Rats were anesthetized with an intraperitoneal injection of pentobarbital and were perfusion fixed with 4% cold paraformaldehyde in phosphate-buffered saline (PBS) at various times (n = 4 for each time point). The eyes were enucleated, cut in half, and fixed with 4% paraformaldehyde in PBS for 30 minutes at 4°C. For subunit p65 of NF-κB, fixed specimens were embedded in paraffin and sectioned at 5 μm. The sections were immunostained with a rabbit avidin-biotin complex (ABC) staining system (Santa Cruz Biosciences). For TNF-α, sections were deparaffinized in xylene and hydrated with graded ethanol. Slides were boiled in target retrieval solution buffer (Dako, Kyoto, Japan) in a microwave for 4 minutes and then placed at room temperature for 20 minutes. Endogenous peroxidase activity in the tissue was quenched with 1% hydrogen peroxide in PBS for 10 minutes. After blocking with 1.5% normal goat serum in PBS, slides were
incubated with rabbit anti-rat NF-κB polyclonal antibody (Santa Cruz Biotechnology) for 1 hour. Biotinylated secondary antibody (goat anti-rabbit IgG) and avidin-biotinylated horseradish peroxidase complex reagent was applied, respectively, for 30 minutes each. Also, 3,3′-diaminobenzidine (Dako) was used as a chromogenic substrate. The sections were counterstained with hematoxylin (Wako Pure Chemicals, Osaka, Japan) and mounted (Aquatex; Merck, Darmstadt, Germany). To verify the binding specificity for NF-κB, some sections were also incubated with normal goat IgG or without the primary antibody (data not shown).

For TNF-α, specimens were OCT embedded, frozen, cryosectioned at 10 μm, and subjected to immunofluorescence staining. After blocking with 10% normal bovine serum in PBS, slides were incubated with goat anti-rat TNF-α polyclonal antibody (Santa Cruz Biotechnology) for 1 hour and immunolabeled with Texas red–conjugated bovine anti-goat IgG secondary antibody for 30 minutes. To verify the binding specificity for TNF-α, some sections were also incubated with normal bovine IgG or without the primary antibody (data not shown).

Statistics
All data are expressed as the mean ± SD. Statistical analysis was performed using the Scheffe procedure for multiple comparisons of means. P < 0.05 was considered statistically significant.

RESULTS

Animals
No marked changes in body weight were noted after injection of LPS and rolipram. A small number of rats treated with 30 μmol/kg rolipram showed mild alterations in behavior including hypoaactivity, forepaw shaking, grooming activity, and head twitches.

Protein and Cells in the Aqueous Humor
In our preliminary study, no elevation of intracameral cells and protein was found after 10 μg of LPS was injected, whereas marked elevation of intracameral cells and protein was observed after 300 μg of LPS was administered. After injection of 100 μg LPS into the rat footpad, protein in the aqueous humor increased at 6 hours, reached maximum levels (21 ± 2.2 mg/mL) at 18 to 30 hours, and then gradually decreased. Rolipram injected 30 minutes before LPS administration inhibited elevation of intracameral protein in a dose-dependent manner (15–30 μmol/kg) 24 hours after LPS administration (Fig. 1). At 30 μmol/kg, rolipram decreased the protein level (10.6 ± 1.4 mg/mL). No cells were found in the aqueous humor of rats treated with 0.9% NaCl (Fig. 2). Cells (35 ± 15 ×
10⁷/mL were present in the aqueous humor of rats 24 hours after LPS injection. Rolipram inhibited the increase in number of cells in the aqueous humor in a dose-dependent manner (15–30 μmol/kg) 24 hours after LPS administration. Rolipram (30 μmol/kg) decreased the number of cells to 5 ± 4 × 10⁵ /mL.

Expression of mRNAs for E-selectin, TNF-α, IL-6, and iNOS after LPS Injection

After injection of LPS into the footpad, mRNAs for E-selectin, TNF-α, and IL-6 in the iris and ciliary body were determined 3 hours after LPS injection, and iNOS mRNA levels were determined 6 hours after LPS injection. Rolipram inhibited the expression of mRNAs for E-selectin (Fig. 3a), TNF-α (Fig. 3b), and IL-6 (Fig. 3c) in the iris and ciliary body 3 hours after LPS administration in a dose-dependent manner (7.5–30 μmol/kg). The mRNA levels for E-selectin, TNF-α, and IL-6 in the iris and ciliary body of the rats treated with 30 μmol/kg rolipram and LPS were 31 ± 3, 10 ± 2, and 444 ± 70-fold, respectively. Rolipram did not inhibit the expression of iNOS mRNA in the iris and ciliary body 6 hours after LPS administration (Fig. 3d).

Inhibition of Expression of mRNAs for E-selectin, TNF-α, IL-6, and iNOS by Rolipram

The levels of mRNAs for E-selectin, TNF-α, and IL-6 in the iris and ciliary body increased at 1.5 hours, reached maximum levels (88 ± 5, 62 ± 8, and 1250 ± 150-fold, respectively) at 3 hours, and then gradually decreased. After injection of LPS into the footpad, iNOS mRNA in the iris and ciliary body increased at 3 hours, reached maximum level (75 ± 11-fold) at 6 hours, and then gradually decreased.

Inhibition of Protein Levels of TNF-α and IL-6 in the Anterior Chamber by Rolipram

After injection of LPS into the footpad, protein levels of TNF-α and IL-6 in the anterior chamber increased, reached maximum levels (350 ± 30 and 1239 ± 123 pg/mL, respectively) at 4 to 6 hours, and then gradually decreased. Rolipram (30 μmol/kg) decreased TNF-α (Fig. 4a) and IL-6 (Fig. 4b) (63 ± 7 and 401 ± 43 pg/mL, respectively) in the anterior chamber 4 hours after LPS administration.

Effect of Rolipram on Nitrite Levels in the Aqueous Humor after LPS Injection

After injection of LPS into the footpad, nitrite levels in the aqueous humor increased, reached maximum level (12.65 μM) at 14 to 20 hours, and then gradually decreased. Rolipram did not inhibit nitrite levels in the aqueous humor 6 hours after administration of LPS (Fig. 4c).

Immunohistochemistry of NF-κB p-65 in the Iris-Ciliary Body after LPS Injection

After injection of LPS into the footpad, activated NF-κB p65 immunoreactivity on the nuclear region of cells in iris-ciliary body was strong at 1 hour (Fig. 5). Weak immunoreactivity was found 4 hours after LPS. One hour after LPS injection, rolipram at 15 and 30 μmol/kg decreased the number of NF-κB p65-positive cells in the iris-ciliary body.

DISCUSSION

In the present study, protein and cells in the anterior chamber increased 18 to 30 hours after 100 μg of LPS was injected. Our findings of LPS-induced anterior uveitis in Wistar rats were similar to those in Lewis and Wistar rats described by Rosenbaum et al., and in young Lewis rats described by Hoekzema et al.^2^ In our preliminary study, rolipram was injected intraperitoneally 60 or 30 minutes before, at the same time of, or 30 hours after LPS injection, rolipram at 15 and 30 μmol/kg decreased reactivity.
minutes after administration of LPS. Rolipram injected 30 minutes before LPS was most effective in inhibiting LPS-induced elevation of intracameral protein and cells. In our present study, therefore, rolipram was injected 30 minutes before LPS.

Alterations in behavior occurred in some rats treated with rolipram in the present study. The alterations were mild and were observed only in a small number of rats treated with 30 μmol/kg doses. Our findings were similar to those described by Wachtel.25

Hoekzema et al.2 reported that after LPS injection, the IL-6 concentration increased in the aqueous humor of Lewis rats. In a study by de Vos et al.3 TNF-α and IL-6 levels in the aqueous humor of Lewis rats were elevated 4 hours after administration of LPS. Suzuma et al.4 have reported observing E-selectin immunoreactivity on the vessel walls of the iris 7 hours after LPS treatment in male Lewis rats. Our present findings of LPS-induced expression of mRNAs for E-selectin, TNF-α, and IL-6 and elevation of TNF-α and IL-6 in the aqueous humor in Wistar rats were similar to those in Lewis rats described by these investigators.2–4 Goureau et al.26 reported that after LPS injection, iNOS mRNA in the iris-ciliary body increased at 2 to 24 hours, and nitrite in the aqueous humor increased in Lewis rats to 18 μM at 16 hours. Increased expression of iNOS mRNA 6 hours after LPS injection and elevated nitrite production 16 hours after LPS injection in our present study were similar. Baeuerle and Henkel11 reported that LPS induces translocation of NF-κB and mediates the overproduction of TNF-α. In our present study, NF-κB immunoreactivity was strong 1 hour after LPS injection. Ollivier et al.25 reported that cAMP inhibited NF-κB-mediated transcription in human monocyteic cells and endothelial cells. Our findings of LPS-induced translocation of NF-κB and its inhibition by rolipram are compatible with those findings.11,26

Rolipram suppressed several types of inflammation.16–19 Klemm et al.17 reported that rolipram inhibited endogenous TNF-α production in a murine model of acute inflammation induced by zymosan. In a study by Buttini et al.18 the level of TNF-α mRNA induced in rat brain by LPS challenge was reduced by intraperitoneal administration of rolipram. Ross et al.19 have proposed that a major mechanism of action of rolipram in collagen-induced arthritis is suppression of TNF-α activity. In our present study, rolipram inhibited LPS-induced anterior uveitis and suppressed mRNAs for E-selectin, TNF-α, and IL-6 and protein levels of TNF-α and IL-6 in Wistar rats.

Increased NO production has been thought to be involved in LPS-induced uveitis.7–25 In our present study, increased nitrite production was found in LPS-induced uveitis. Also, rolipram decreased LPS-induced elevation of protein and cells in the aqueous chamber, did not inhibit the expression of iNOS mRNA, and increased nitrite production induced by LPS. Dutta et al.27 reported that the injection of LPS increased iNOS activity in the lung of Long-Evans rats, but pretreatment with rolipram did not affect NOS activity. Ross et al.19 reported that rolipram inhibited the expression of TNF-α and IL-12, but did not affect NO production in collagen-induced arthritis in mice.

![Figure 5](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932928/) Immunohistochemistry of NF-κB p65 in the iris-ciliary body of the rat after injection of LPS. (a) NaCl (0.9%) injection. One hour after injection of (b) LPS, (c) LPS and 15 μmol/kg rolipram, or (d) LPS and 30 μmol/kg rolipram. Brown reaction products indicate positive immunoperoxidase localization of NF-κB p65. Results are representative of four experiments. Light hematoxylin staining; original magnification, ×400.

![Figure 6](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932928/) Immunohistochemistry of TNF-α in the iris-ciliary body of the rat after injection of LPS. (a) NaCl (0.9%) injection. Four hours after injection of (b) LPS, (c) LPS and 15 μmol/kg rolipram, or (d) LPS and 30 μmol/kg rolipram. Fluorescein (arrows) indicates localization of TNF-α. Results are representative of four experiments. Original magnification, ×400.
Lieber et al.,26 reported that rolipram showed no inhibitory effect on LPS-induced iNOS or NO synthesis. The effects of rolipram on NO production that we observed were similar to those described previously,26,27,28 It is unclear at present whether rolipram is clinically useful in treating uveitis or not. In conclusion, the findings in our present study indicate that NF-kB translocation and expression of E-selectin, TNF-α, and IL-6 were involved in the pathogenesis of LPS-induced uveitis and were inhibited by rolipram. In contrast, the inhibitory effect of rolipram on LPS-induced uveitis was independent of iNOS synthesis and NO production in Wistar rats.

References

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