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Molecular Mechanisms of Topical Anti-Inflammatory Effects of Lipoxin A_4 in Endotoxin-Induced Uveitis[S]

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ABSTRACT

Lipoxin A_4 (LXA_4) is a lipid mediator that plays an important role in inflammation resolution. We assessed the anti-inflammatory effect of LXA_4 on endotoxin-induced uveitis (EIU) in rats. The inflammatory cell number and levels of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), prostaglandin E_2 (PGE_2), and protein, as well as expression of cyclooxygenase-2 (COX-2) and vascular endothelial growth factor (VEGF), in the anterior chamber of the eye were determined 24 h after lipopolysaccharide (LPS; 200 μg/paw) intradermal injection. The immunohistochemical reactivities of nuclear factor-κB (NF-κB) and c-Jun were also examined. Topical LXA_4 (1–10 ng/eye) pretreatment decreased the number of inflammatory cells and the protein leakage into the aqueous humor (AqH). In addition, topical LXA_4 (10 ng/eye) inhibited the LPS-induced production of IL-1β, TNF-α, and PGE_2, and expression of COX-2 and VEGF. A decreased activation of NF-κB and c-Jun was also found in LXA_4-treated eyes. It is very interesting that an anti-inflammatory effect was achieved even when LXA_4 (10 ng/eye) was applied topically after LPS challenge, as indicated by the reduction in the cellular and protein extravasations into the AqH. Moreover, topical treatment of corticosteroid prednisolone (200 μg/eye) beginning before or after LPS injection reduced all of the molecular and biochemical alterations promoted on EIU rats in an efficacy similar to that of LXA_4. Together, the present results provide clear evidence that pharmacological activation of LXA_4 signaling pathway potently reduces the EIU in rats. Therefore, LXA_4 stable analogs could represent promising agents for the management of ocular inflammatory diseases.

Uveitis is one of the most harmful ocular conditions in humans. It can affect any part of the eye, and its recurrent nature may result in secondary complications such as cataract, cystoid macular edema, glaucoma, and, ultimately, destruction of the intraocular tissues and blindness. Uveitis may be caused by infectious organisms or by an immune-mediated process, including Behçet's disease, ankylosing spondylitis, juvenile rheumatoid arthritis, Reiter's syndrome, and inflammatory bowel disease (Durrani et al., 2004). Present pharmacological treatment for uveitis includes corticosteroids, chemotherapeutic agents, and tumor-necrosis factor inhibitors to diminish inflammation. Nevertheless, lack of patient and/or disease responsiveness, resistance to long-term treatment, and severe side effects from these drugs limit their use (Dunn, 2004). Given these restrictions, there is an obvious demand for the development of new therapeutic strategies.

Recent advances in knowledge of the mechanisms of inflammatory resolution and the discovery of several endogenous antiinflammatory mediators has led to a whole new range of potential therapeutic possibilities (Serhan, 2007). Lipoxins are the first endogenous lipid mediators with anti-inflammatory properties involved in the inflammatory resolution process to have been described previously (Serhan et al., 1984). Lipoxin A_4 (LXA_4) biosynthesis is a transcellular process in which the interaction of different cell types, such as leukocytes, platelets, and vascular endothelium, by means of adhesion molecules, plays an important role. LXA_4 bind with high affinity to a G protein-coupled receptor termed

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ABBREVIATIONS: LXA_4, lipoxin A_4; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; PGE_2, prostaglandin E_2; COX, cyclooxygenase; VEGF, vascular endothelial growth factor; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; AqH, aqueous humor; EIU, endotoxin induced uveitis; i.pl., intraplantar; PBS, phosphate-buffered saline; ICB, iris ciliary body; AP-1, activator protein-1.

LXA_4 receptor (ALXR), also known as FPRL1 and FPR2 (Chiang et al., 2006). It is noteworthy that recently the rat ALXR has been cloned from peripheral blood leukocytes, and it presented a homology of approximately 74 and 84% to the deduced amino acid sequences of the human and mouse ALXR, respectively (Chiang et al., 2003). Activation of ALXR by LXA_4 leads the reduction of many endogenous processes such as leukotriene function, leukocyte migration, tumor necrosis factor (TNF)-induced production of chemokines, expression of chemokine receptors and adhesion molecules, pathogen-triggered production of interleukin (IL)-12, and superoxide formation (Serhan, 2007). Likewise, evidence has shown that LXA_4 signaling also attenuates nuclear factor-κB (NF-κB) activation and blocks phosphorylation of p38, extracellular signal-regulated kinase, and c-Jun N-terminal kinase (József et al., 2002; Svensson et al., 2007). It is noteworthy that these intracellular signaling pathways have been identified recently as being involved in the pathophysiology of uveitis.

The role of LXA_4 in ocular tissues and diseases has been investigated in recent years. For instance, Gronert and colleagues (1998) have reported the expression of ALXR mRNA in human corneas, providing the first evidence for a potential specific bioaction of LXA_4 in the eye. Extending these data, experiments in mice have demonstrated that LXA_4 and the enzyme 12/15-LOX have been found in healthy and wounded corneas, whereas in contrast, 12/15-LOX-deficient mice cannot promote normal wound healing. In addition, the ALXR in healthy corneas is predominantly expressed in epithelial cells as de-epithelialization abrogates not only 12/15-LOX but also ALXR mRNA expression. It has been reported that topical LXA_4 within the corneal tissue promotes wound healing and is capable of reducing tissue damage after injury (Gronert et al., 2005). A recent study has provided experimental support indicating that 12/15-LOX and heme-oxygenase systems function in concert to control inflammatory response in the cornea (Biteman et al., 2007). Finally, LXA_4 has been shown to lower intraocular pressure in rabbits (Serhan, 2007). However, despite the key role of LXA_4 in regulating most inflammatory and reparative responses in the cornea, to date, little is known on the LXA_4 effect in the intraocular inflammatory diseases. For this reason, the present study was designed to investigate the possible anti-inflammatory effect of topical LXA_4 administration on several well-described inflammatory markers in endotoxin induced uveitis (EIU) in rats. Data obtained provide the first evidence showing that topical LXA_4 application significantly stimulated the reduction of inflammatory process in the anterior chamber of the eye.

Materials and Methods

Animals. Nonfasted male Wistar rats (6 weeks old, 140–180 g) kept in controlled temperature (22 ± 2°C) and humidity (60–80%) under a 12-h light/dark cycle (lights on at 6:00 AM) were used. All experiments were conducted in compliance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Ethics Committee of the Universidade Federal de Santa Catarina.

Induction of EIU and Drug Treatment Protocol. The EIU was induced by an intraplantar (i.pl.) injection of 0.1 ml of saline containing 200 μg of LPS from *Escherichia coli* (serotype 0111:B4, Sigma-Aldrich, São Paulo, Brazil) into one rat hind paw. Control animals received the same volume of pyrogen-free saline intradermally (control group). The LXA_4 [1, 5, or 10 ng/eye; (5*S*,6*R*,15*S*)-trihydroxy-7,9,13-*trans*-1-*cis*-eicosatetraeonic acid; Cayman Chemical, Ann Arbor, MI] was applied topically (20-μl eye drops) to both rat eyes 1 h before and 6, 12, and 18 h after LPS administration. A separate group of animals was treated with pyrogen-free saline (topical vehicle group). As a positive control, some animals received topical applications of corticosteroid prednisolone (200 μg/eye; Allergan, São Paulo, Brazil) at the same time points as LXA_4. In another set of experiments, to evaluate the therapeutic efficacy as a postinflammatory treatment, LXA_4 (10 ng/eye) was applied topically (20-μl eye drops) to both eyes 6, 12, and 18 h after LPS administration. A separate group of animals was treated with saline (vehicle) or with prednisolone (200 μg/eye) at the same time points. All animals were anesthetized with isoflurane (1%; Abbot Laboratórios do Brasil Ltda., Rio de Janeiro, Brazil) using a vaporizer system (SurgiVet Inc., Waukesha, WI) before all experimental manipulations.

Quantification of Infiltrating Cells and Protein Concentration in Aqueous Humor. Aqueous humor (AqH, 20–25 μl/rat) was collected from both eyes by an anterior chamber puncture using a 29-gauge needle as described previously (Rodrigues et al., 2007). For cell counting, the AqH sample was diluted in Türk stain solution (1:20), and the cells were counted with a hemocytometer under a light microscope. The number of cells per field was counted manually, and the number of cells per microliter was obtained by averaging the results of four fields from each sample. The total protein concentration in the AqH samples was measured using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions.

Quantification of IL-1β, TNF-α, and PGE_2 Levels in AqH. The levels of IL-1β and TNF-α in the AqH obtained from rats with EIU were assessed with a commercially available enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN), whereas the levels of PGE_2 were assessed with a commercially available EIA kit (Cayman Chemical) according to the manufacturer's instructions. The AqH from the eyes of two to three rats was pooled, and 50 μl was used for a single assay. All measurements were performed in duplicate.

Histopathological and Immunohistochemical Studies. Rat eyes were collected and fixed in a PBS solution containing 4% paraformaldehyde and 0.2% glutaraldehyde (0.2 ml of 25% stock per 25 ml) for 24 h at room temperature, dehydrated by graded ethanol, and embedded in paraffin. For histological evaluation, tissue sections (5 μm) were deparaffinized with xylene and stained using hematoxylin and eosin. Infiltrating inflammatory cells in the iris ciliary body (ICB) and iris were evaluated upon visual inspection in a masked fashion using a counting grid at 400× magnification. The number of infiltrating inflammatory cells in six sections per eye in the ICB and in three sections per eye in the iris was averaged and recorded.

Immunohistochemical detection of vascular endothelium growth factor (VEGF), cyclooxygenase-2 (COX-2), p65 NF-κB, and c-Jun AP-1 were assessed in the ICB and/or iris (5 μm slices) using polyclonal rabbit anti-VEGF (1:200; Santa Cruz Biotechnology Inc., Santa Cruz, CA), polyclonal rabbit anti-COX-2 (1:50), polyclonal rabbit anti-phospho-p65 NF-κB (1:50), and polyclonal rabbit anti-phospho-c-Jun (1:50) (all from Cell Signaling Technology, Danvers, MA). High-temperature antigen retrieval was performed by immersion of the slides in a water bath at 95 to 98°C in 10 mM trisodium citrate buffer, pH 6.0, for 45 min. The nonspecific binding was blocked by incubating sections for 1 h with goat normal serum diluted in PBS. After overnight incubation at 4°C with primary antibodies, the slides were washed with PBS and incubated with the secondary antibody Envision plus (Dako North America, Inc., Carpinteria, CA), ready-to-use, for 1 h at room temperature. The sections were washed in PBS, and the visualization was completed by use of 3,3′-diaminobenzidine (Dako North America, Inc.) in chromogen solution and counterstained lightly with Harris's hematoxylin solution. Images were obtained with a microscope (Nikon Eclipse

50i) and Digital Sight Camera (DS-5M-L1; both from Nikon, Melville, NY). Control and experimental tissues were placed on the same slide and processed under the same conditions. Settings for image acquisition were identical for control and experimental tissues. For each rat eye, six images (one per section) of the ICB and three images (one per section) of the iris were obtained. Digitized, eight-bit images were transferred to a computer, and the average pixel intensity of VEGF and COX-2 staining was calculated for each image using NIH ImageJ 1.36b imaging software (http://rsb.info.nih.gov/ij/). For each rat eye, the values obtained for the ICB or the iris tissues were averaged. Phospho-p65 NF-κB or phospho-c-Jun-positive cells were determined upon visual inspection in the same eye areas in a masked fashion using a counting grid at 400$\times$ magnification and expressed as the percentage of positive cells.

Statistical Analysis. Data are expressed as mean $\pm$ S.D. of at least three independent experiments. The results were analyzed by one-way analysis of variance followed by Bonferroni's post hoc test for multiple comparisons, and values of $P < 0.05$ were considered significant. All tests were carried out using the Statistica software package (StatSoft Inc., Tulsa, OK).

The term "control" refers to animals that received only an intradermal injection of sterile saline into the hind paw. All other groups received an intradermal injection of LPS into the hind paw. Results from topically LXA_4- or prednisolone-treated animals were compared with the respective vehicle-treated animals.

Results

LXA_4 Stimulates the Reduction of Inflammatory Cell Migration and Protein Leakage into the AqH during EIU. The visual axis of the eye, which must focus light images precisely on the retina, is extremely delicate and intolerant of the distortion that often accompanies inflammation. Any disturbance in homeostasis of the eye can lead to biological malfunctioning and/or diffraction, absorbance, or reflection of photons, resulting in disturbed or diminished vision (Streilein, 2003). For this reason, under homeostatic conditions, a very low level of soluble proteins (1.1 ± 0.3 mg/ml) and no inflammatory cells were found in the anterior chamber of the eye. On the other hand, the EIU was characterized by a time-dependent increase in the protein levels and the number of inflammatory cells into the eye. The inflammation in response to LPS administration was observed as early as 6 h, reaching the maximal response at 24 h and persisting until at least 72 h later (Supplemental Fig. 1). The protein levels and the number of inflammatory cells in the AqH of LPS-treated animals 24 h after injection were 23 ± 0.9 mg/ml and $53 \pm 9 \times 10^5$ cells/ml, respectively (Fig. 1). Next, we attempted to outline the potential LXA_4 anti-inflammatory effect in the EIU. Data shown in Fig. 1, A and B, indicate that the level of soluble proteins and the number of inflammatory cells were dose-dependent and significantly reduced in animals pretreated topically with LXA_4 compared with topically vehicle-treated animals. The calculated reductions over LPS-induced protein leakage and cellular influx caused by LXA_4 (10 ng/eye) pretreatment were 60 and 70%, respectively. Topical pretreatment with prednisolone (200 μg/eye, used as positive control drug) also significantly reduced the cellular infiltration and protein leakage into the AqH compared with topically vehicle-treated animals (inhibitions of 90 and 72%, respectively).

In another set of experiments, to evaluate the therapeutic efficacy of topically administered LXA_4 as a postinflammatory treatment, we began treating animals with LXA_4 only 6 h after LPS challenge (Fig. 2). At this time point, alterations in the vascular permeability in the EIU-animals, mainly protein and cell extravasations, were found to be distinct from those of the control animals (Supplemental Fig. 1). It is noteworthy that topical LXA_4 (10 ng/eye) after treatment induced a significant reduction of vascular changes found in EIU compared with vehicle-treated animals. The decrease over LPS-induced protein leakage and cellular influx stimulated by LXA_4 (10 ng/eye) after treatment were 50 and 63%, respectively. Likewise, topical (200 μg/eye) after treatment also reduced the LPS-induced protein and cellular extravasations into the AqH compared with vehicle-treated animals (inhibitions of 74 and 83%, respectively). These results provide new insights into the EIU model, revealing LXA_4 as a relevant therapeutic alternative for the treatment of ocular inflammatory diseases.

LXA_4 Reduces Histopathological Changes during EIU in the ICB and Iris. Histological analysis revealed no infiltrating cells in the eyes of animals that had received an intradermal injection of sterile saline into the hind paw (control animals). On the other hand, the EIU was characterized by a massive neutrophil infiltration into the eye when assessed 24 h after LPS injection, mainly into the ICB (18 ± 7 cells/section; Fig. 3A) but also into the iris (5 ± 3 cells/section; Fig. 3B). Topical pretreatment with LXA_4 (10 ng/eye) induced a significant decrease in the number of inflammatory cells in the anterior chamber of the eye compared with topical vehicle-treated animals. On average, 6 ± 4 and 2 ± 1 inflammatory cells per ocular section were detected after topical pretreatment with LXA_4 (10 ng/eye) in the ICB and iris during EIU, respectively. A similar inhibition was found for topical pretreatment with prednisolone (200 μg/eye) (Supplemental Fig. 2).

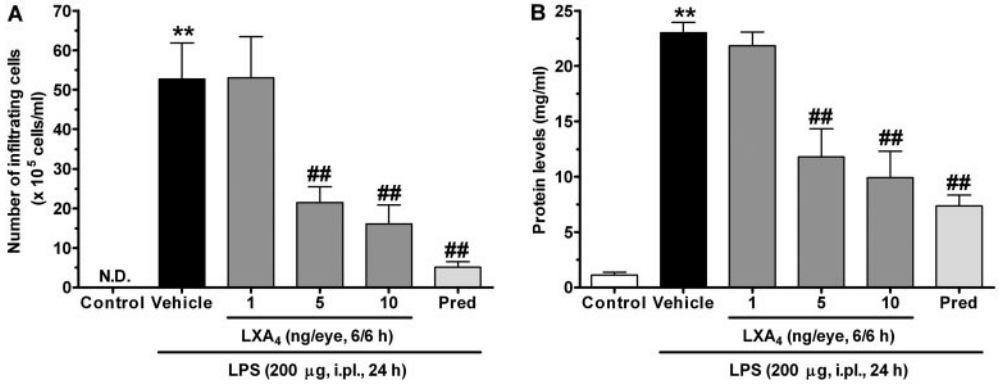

Fig. 1. Effect of LXA_4 on cellular and protein extravasations into the AqH during EIU. Vehicle (saline), LXA_4 (1, 5, or 10 ng/eye) or prednisolone (200 μg/eye) was applied topically (20-μl eye drops) to both eyes 1 h before and 6, 12, and 18 h after LPS (200 μg, i.pl.) administration. Cell count (A) and protein levels (B) were assessed in the AqH 24 h after EIU. The values represent the mean $\pm$ S.D. ($n = 8$/group). **, $P < 0.01$ compared with control group (saline, i.pl.), and ##, $P < 0.01$ compared with vehicle-treated group. Pred, prednisolone; N.D., not detected.

The Effect of LXA_4 on TNF-α and IL-1β Levels in the AqH during EIU. Cytokines are multifunctional molecules that play an important role in host defense, acute-phase reactions, immune response, and hematopoiesis. Their productions is up-regulated by various factors, including bacterial endotoxin LPS (O'Shea et al., 2002). The results depicted in Fig. 4 indicate that undetectable or very low levels of cytokines were found in saline-treated animals (control group). As expected, up-regulated levels of TNF-α and IL-1β were present in rat AqH 24 h after LPS treatment (224 $\pm$ 40 and 1120 $\pm$ 252 pg/ml, respectively). When administered topically, LXA_4 (10 ng/eye) significantly reduced the augmentation of both inflammatory cytokines in response to LPS compared with topical vehicle-treated animals. The inhibitions obtained in the levels of TNF-α and IL-1β were 95 and 96%, respectively. A similar effect was verified in the group pretreated topically with prednisolone (inhibitions of 92 and 98% for TNF-α and IL-1β, respectively).

LXA_4 Reduced PGE_2 Levels during EIU through Inhibition of COX-2 Expression. High levels of prostaglandins are produced by COX enzymes during most inflammatory responses (Turini and DuBois, 2002). Upon LPS administration, the production of PGE_2 increased significantly in the AqH with respect to the control group 24 h after challenge (Fig. 5A). Topical LXA_4 (10 ng/eye) pretreatment significantly inhibited the increase of PGE_2 production compared with topical vehicle-treated animals. The percentage of inhibition obtained for LXA_4 was 75%. In addition, when the animals were pretreated with the positive control drug prednisolone (200 μg/eye), a significant inhibition in PGE_2 increase was found (inhibition of 82%). To gain further insights into the mechanisms involved in the inhibitory effects of LXA_4 on PGE_2 production after EIU, we evaluated the possible influence of this compound on COX-2 expression (Fig. 5, B and C). An augmentation in the expression of COX-2 in the anterior chamber of the eye in the EIU has been shown previously (Yadav et al., 2007). Supporting these data, immunohistochemical analysis revealed that EIU resulted in a dramatic up-regulation of COX-2 protein expression in the ICB (10-fold) and iris (6.5-fold). It is noteworthy that topical application of the same dose of LXA_4, which was effective in inhibiting the increase of PGE_2 levels after EIU, caused a significant suppression of the COX-2 protein expression in both ocular tissues. The inhibitions obtained were 80 and 84% in the ICB and iris, respectively. Topical treatment with prednisolone (200 μg/eye) also significantly reduced the expression of COX-2 during EIU (inhibitions of 88 and 92% in the ICB and iris, respectively) (Supplemental Fig. 3).

LXA_4 Reduces the Increase in VEGF Expression in the ICB during EIU. In addition to its role in promoting endothelial permeability and proliferation, a growing body of evidence has highlighted the contribution of VEGF in inflammation. To assess the VEGF levels in the eye after EIU, we performed the immunohistochemical analyses. The data depicted in Fig. 6 indicate that very low levels of VEGF were found in the control animals. On the other hand, systemic administration of LPS to rats resulted in increased VEGF protein levels at 24 h in the ICB (7.3-fold). When rats were pretreated topically with LXA_4 (10 ng/eye), the VEGF expression during EIU was markedly reduced (76%). A similar inhibition was found for topical pretreatment with prednisolone (200 μg/eye) (Supplemental Fig. 4). These data provide additional information concerning the effect of lipoxins over VEGF functions.

Suppression of NF-κB and AP-1-Mediated Transcriptional Induction during EIU by LXA_4. The two major signaling systems activated by inflammatory stimuli involve the transcriptional factors NF-κB and AP-1 that modulate

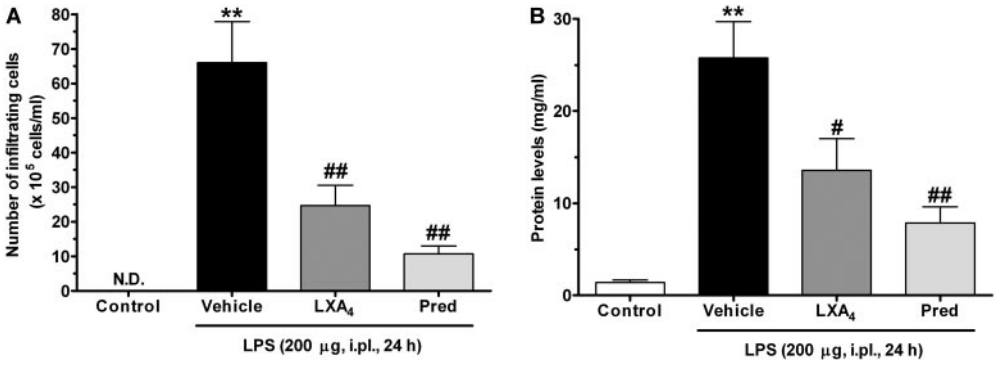

Fig. 2. LXA_4 after LPS injection inhibits cellular migration and protein leakage during EIU. Vehicle (saline), LXA_4 (10 ng/eye), or prednisolone (200 μg/eye) was applied topically (20-μl eye drops) to both eyes, 6, 12, and 18 h after LPS (200 μg, i.pl.) administration. Cell count (A) and protein levels (B) were assessed in the AqH 24 h after EIU. The values represent the mean $\pm$ S.D. ($n = 8$ per group). **, $P < 0.01$ compared with control group (saline, i.pl.); #, $P < 0.05$ or ##, $P < 0.01$ compared with vehicle-treated group. Pred, prednisolone; N.D., not detected.

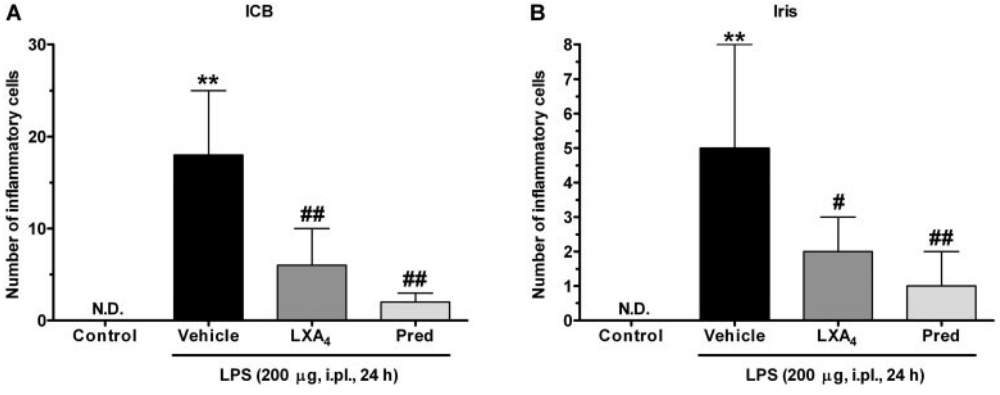

Fig. 3. Effect of LXA_4 on histological changes in the anterior chamber of the eye during EIU. Vehicle (saline), LXA_4 (10 ng/eye), or prednisolone (200 μg/eye) was applied topically (20-μl eye drops) to both eyes 1 h before and 6, 12, and 18 h after LPS (200 μg, i.pl.) administration. The number of infiltrating inflammatory cells in six sections per eye in the ICB (A) and in three sections per eye in the iris (B) 24 h after EIU was averaged and recorded. The values represent the mean $\pm$ S.D. ($n = 8$ per group). **, $P < 0.01$ compared with control group (saline, i.pl.); #, $P < 0.05$ or ##, $P < 0.01$ compared with vehicle-treated group. Pred, prednisolone; N.D., not detected.

the expression of several genes implicated in the control of inflammatory responses. It has been shown that p65 NF-κB and c-Jun AP-1 subunit phosphorylation is necessary for the transcriptional competence of both transcriptional factors (Ghosh and Karin, 2002). To assess the phosphorylation state of p65 NF-κB and c-Jun, we used the technique of immunohistochemistry (Fig. 7). Under homeostatic conditions, no phosphorylated p65 NF-κB or c-Jun was found in the ICB and iris. EIU, as expected, induced marked phosphorylation of p65 NF-κB and c-Jun and their subsequent translocation to the nucleus in the cells of ICB and iris, which peaked at 6 h and remained significantly increased up to 12 h after LPS administration. The activation of both proteins was found to be diminished after 24 h (results not shown). It is interesting that topical LXA_4 (10 ng/eye) pretreatment significantly reduced the activation of p65 NF-κB and c-Jun in the anterior chamber of the eye compared with the vehicle-treated group. The inhibitions in the p65 NF-κB activation in the ICB and iris after EIU by LXA_4 were 63 and 64%, respectively. Likewise, LXA_4 significantly diminished the c-Jun activation during EIU by 64 and 66% in the ICB and iris, respectively. Topical treatment with prednisolone (200 μg/eye) also reduced the activation of p65 NF-κB and c-Jun AP-1 during EIU (Supplemental Fig. 5).

Discussion

The present study provides the first functional and molecular evidence indicating that activation of LXA_4 signaling pathway greatly attenuates EIU. It is interesting that both pre- and post-topical schedules of treatment revealed that LXA_4 had preventive and therapeutic effects on the EIU. LXA_4 showed an important effect on the inflammatory cascade as indicated by the decrease in the number of infiltrating inflammatory cells and in the protein leakage into the anterior chamber of the eye. The anti-inflammatory effects of LXA_4 seem to be associated with its ability to stimulate the suppression of NF-κB and c-Jun activation, as well as the reduction of TNF-α, IL-1β, COX-2, and VEGF production and/or expression in the anterior chamber of the eye induced by LPS. Moreover, another important finding of our study is the great potency of LXA_4 compared with prednisolone. LXA_4 reduced the EIU in an efficacy similar to that of corticosteroid prednisolone in a dose 20-fold lower.

The host response to pathogenic insults involves complex inflammatory responses and cellular immune reactions. In the ocular tissue, exposure to exogenous bacterial toxins such as LPS is known to induce the breakdown of the blood-aqueous barrier that leads to cellular infiltration and plasma protein extravasation into the AqH (Rosenbaum et al., 1980). In the present study, we demonstrated that activation of LXA_4 signaling pathway reduces the cellular migration and protein leakage into the AqH during EIU. It is interesting that an anti-inflammatory effect was achieved even when LXA_4 was administered topically after LPS injection, indicating an important aspect of its therapy. To our knowledge, this is the first evidence indicating that LXA_4 presents therapeutic properties in an inflammatory process in the anterior chamber of the eye. It is well known that lipoxins regulate the extravascular accumulation of leukocytes during acute inflammation by altering leukocyte-endothelial interaction, as well as having a direct effect on leukocyte diapedesis and chemotaxis (Serhan, 2007). For instance, LXA_4 stable ana-

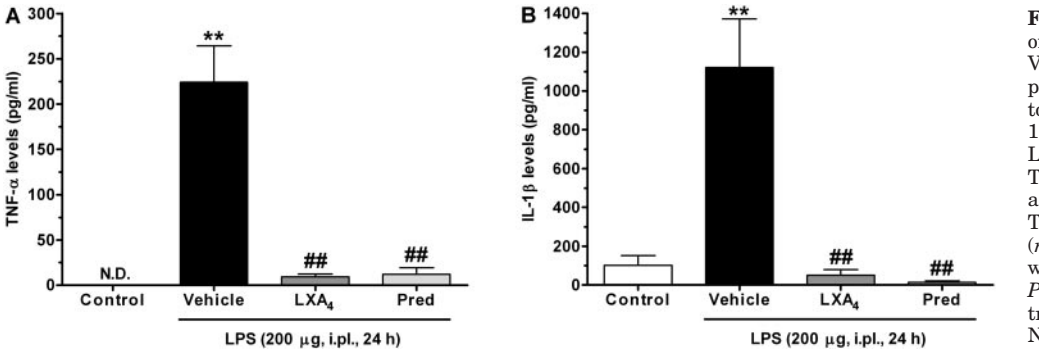

Fig. 4. LXA_4 prevents the production of cytokines in the AqH during EIU. Vehicle (saline), LXA_4 (10 ng/eye), or prednisolone (200 μg/eye) was applied topically (20-μl eye drops) to both eyes 1 h before and 6, 12, and 18 h after LPS (200 μg, i.pl.) administration. TNF-α (A) and IL-1β (B) levels were assessed in the AqH 24 h after EIU. The values represent the mean ± S.D. (n = 5/group). **, $P < 0.01$ compared with control group (saline, i.pl.), ##, $P < 0.01$ compared with vehicle-treated group. Pred, prednisolone; N.D., not detected.

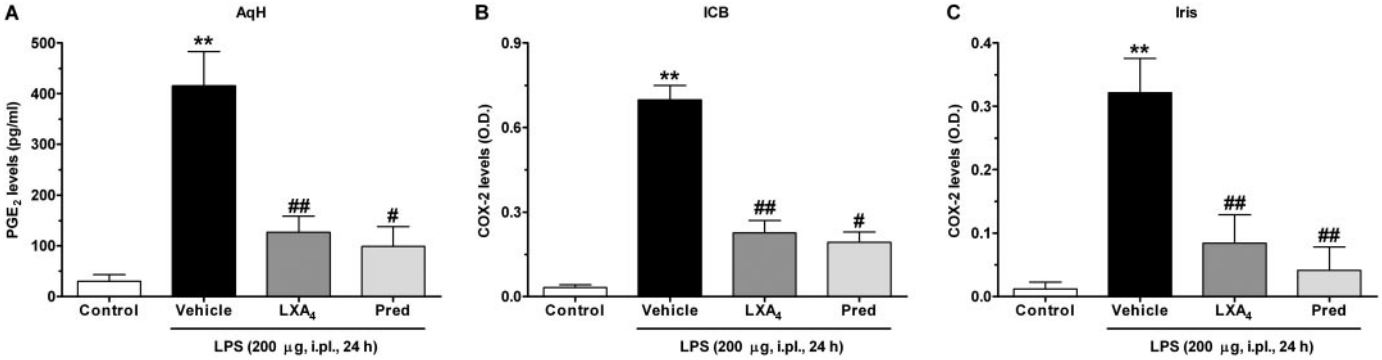

Fig. 5. LXA_4 inhibits PGE_2 production and COX-2 expression in the anterior chamber of the eye during EIU. Vehicle (saline), LXA_4 (10 ng/eye), or prednisolone (200 μg/eye) was applied topically (20-μl eye drops) to both eyes 1 h before and 6, 12, and 18 h after LPS (200 μg, i.pl.) administration. A, PGE_2 levels in the AqH and the expression of COX-2 in the ICB (B) or iris (C) were assessed 24 h after EIU. The values represent the mean ± S.D. (n = 5 per group). **, $P < 0.01$ compared with control group (saline, i.pl.); #, $P < 0.05$ or ##, $P < 0.01$ compared with vehicle-treated group. Pred, prednisolone; O.D., optic density.

logs modulate the expression of some adhesion molecules such as L-/P-selectin and CD11/CD18 (Filep et al., 1999). It is noteworthy that the importance of a range of adhesion molecules to the outcome of EIU has been investigated previously. P-/E-selectin, β2-integrin, and intercellular adhesion molecule-1 have been found to be involved in the ocular inflammatory response to endotoxin (Whitcup et al., 1992; Suzuma et al., 1997, 1998). In addition, it has been shown that LXA_4 and its analogs not only present inhibitory action over granulocyte migration but are also capable of inhibiting the increase in vascular permeability triggered by these cells (Bandeira-Melo et al., 2000; Gronert et al., 2001). Exactly because of this ability of granulocytes to interact with the vasculature, increasing the permeability and contributing to edema formation, lipoxin effects on plasma leakage have commonly been considered as an indirect consequence of the reduction of neutrophil adhesion and influx induced by this lipid mediator in different models. In this context, we have demonstrated previously that LXA_4 presents a marked antiedematogenic action, which seems not to discriminate among different inflammatory stimuli, including bradykinin, carrageenan, platelet-activating factor, and PGE_2. However, we have also shown that LXA_4 stimulates the inhibition of the edematogenic response caused by histamine, which is a mediator typically described as a direct inducer of increased vascular permeability and edema but does not promote leukocyte influx (Menezes-de-Lima et al., 2006). This indicates that the antiedematogenic action of LXA_4 is not simply a consequence of its ability to inhibit granulocyte influx, because even in a model in which edema occurs independently of leukocyte accumulation, LXA_4 is still effective in reducing the vascular permeability. Further investigation should provide additional data to elucidate the precise mechanisms through which LXA_4 reduces the vascular permeability and the consequent protein leakage.

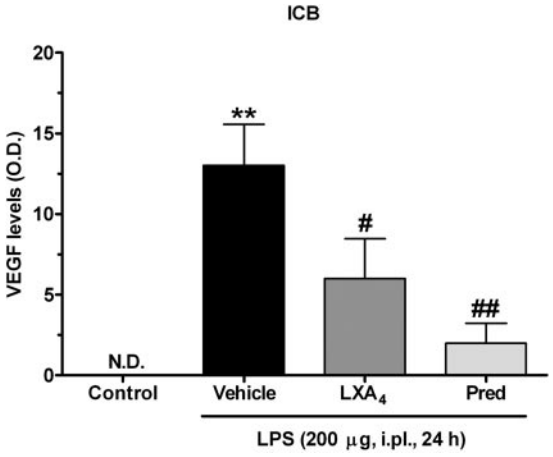

Fig. 6. Effect of LXA_4 on VEGF expression in the ICB during EIU. Vehicle (saline), LXA_4 (10 ng/eye), or prednisolone (200 μg/eye) was applied topically (20-μl eye drops) to both eyes 1 h before and 6, 12, and 18 h after LPS (200 μg, i.pl.) administration. Expression of VEGF in the ICB was assessed 24 h after EIU. The values represent the mean ± S.D. (n = 5/group). **, $P < 0.01$ compared with control group (saline, i.pl.); #, $P < 0.05$ or ##, $P < 0.01$ compared with vehicle-treated group. Pred, prednisolone; N.D., not detected; O.D., optic density.

A central feature of the pathophysiology of acute inflammation triggered by LPS is the production of multiple proinflammatory mediators, including cytokines, chemokines, enzymes, and angiogenic factors (Guha and Mackman, 2001). Reports regarding the effects of cytokines on EIU pathogenesis have indicated that some cytokines, namely IL-1β and

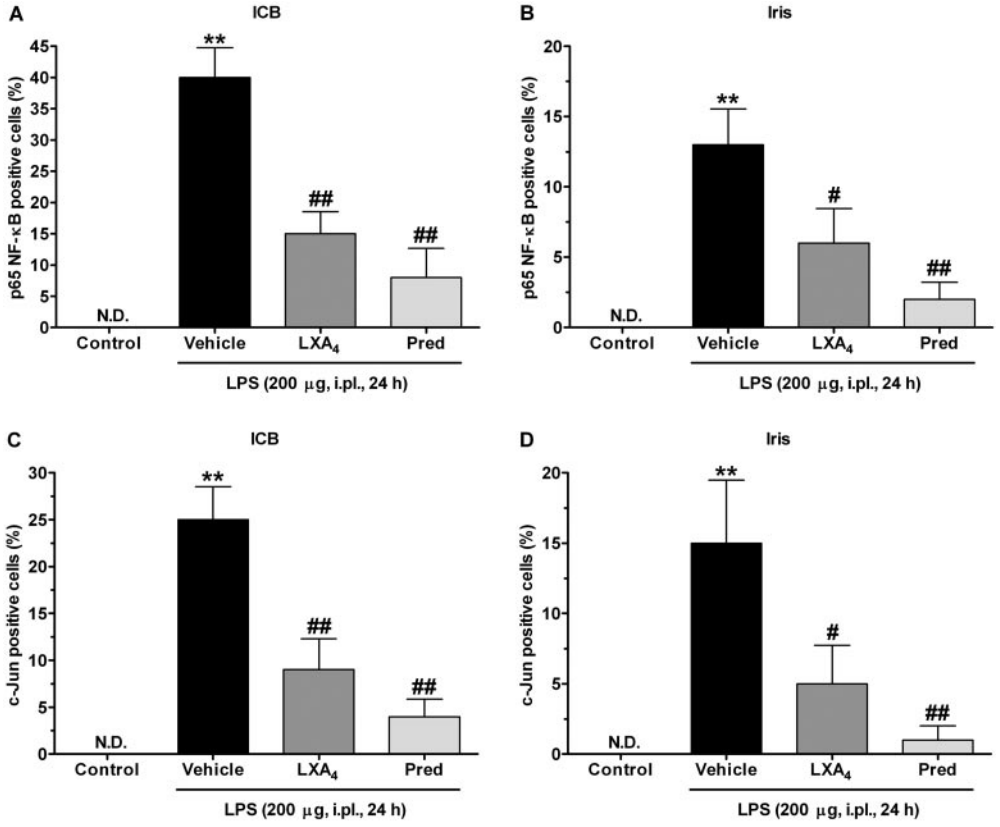

Fig. 7. LXA_4 reduces the activation of p65 NF-κB and c-Jun AP-1 in the anterior chamber of the eye during EIU. Vehicle (saline), LXA_4 (10 ng/eye), or prednisolone (200 μg/eye) was applied topically (20-μl eye drops) to both eyes 1 h before LPS (200 μg, i.pl.) administration. Activation of p65 NF-κB in the ICB (A) and iris (B) or c-Jun in the ICB (C) and iris (D) was assessed 6 h after EIU. Phospho-p65 NF-κB or phosphor-c-Jun-positive cells were determined upon visual inspection in the ICB and iris using a counting grid at 400× magnification and expressed as the percentage of positive cells. The values represent the mean ± S.D. (n = 5 per group). **, $P < 0.01$ compared with control group (saline, i.pl.); #, $P < 0.05$ or ##, $P < 0.01$ compared with vehicle-treated group. Pred, prednisolone; N.D., not detected.

TNF-α, are involved in the initiation of uveitis (de Smet and Chan, 2001). It is noteworthy that several recent studies have suggested that LXA_4 opposes the inflammatory property of TNF-α and IL-1β. Indeed, LXA_4 inhibits IL-1β-induced release of IL-6, IL-8, and matrix metalloproteinase-3 in fibroblast-like synoviocytes, antagonizes TNF-α-initiated IL-1β production by neutrophils (Hachicha et al., 1999; Sodin-Semrl et al., 2000) and attenuates TNF-α-stimulated IL-8 and monocyte chemoattractant protein-1 release by colonic cell lines (Gronert et al., 1998; Goh et al., 2001). Moreover, LXA_4 stable analogs are able to inhibit the renal synthesis of IL-1β in experimental ischemic renal injury (Leonard et al., 2002; Kieran et al., 2003; Wu et al., 2005) and to block the extracellular signal-regulated kinase-dependent TNF-α secretion from human T cells (Ariel et al., 2003). In the present study, we clearly confirmed and also largely extended these previous findings by demonstrating for the first time that topical administration of LXA_4 significantly suppresses TNF-α and IL-1β production in the anterior chamber of the eye during EIU.

The well known enzymes COX-1 and COX-2 are responsible for the transformation of arachidonic acid into prostaglandins. In contrast to the constitutive form COX-1, COX-2 is generally, but not exclusively, induced in response to stimulators such as growth factors, cytokines, tissue injury, and ultraviolet radiation (Mitchell and Warner, 2006). COX-2-induced production of prostanoids is often implicated in pathophysiological states of ocular tissues, and its participation in ocular injury and acute inflammation in the EIU is well documented (Rodrigues et al., 2007; Yadav et al., 2007). The present study provides evidence showing that topical application of LXA_4 results in the decrease of PGE_2 levels in the AqH during EIU. Furthermore, we also assessed whether the inhibition of PGE_2 levels after LXA_4 treatment could be associated with its ability to interfere with the COX-2 expression. Our data show clearly that the topical application of LXA_4 significantly stimulated the inhibition of the LPS-induced expression of COX-2 in the ICB and iris tissue.

COX-2 overexpression has been linked to the increased expression of several angiogenic factors, including VEGF (Kuwano et al., 2004). The expression of VEGF is stimulated in response to hypoxia by activated oncogenes and by a variety of cytokines. VEGF induces endothelial cell proliferation, promotes cell migration, and inhibits apoptosis. Likewise, vascular permeability can be regulated by VEGF and by a wide array of inflammatory mediators (Ferrara et al., 2003). In the ocular tissue, VEGF has been considered to be the major regulator of aberrant and excessive blood vessel growth and permeability. An increase of VEGF expression in the retina in EIU has been demonstrated (Ng et al., 2006). Our results confirm and also extend these previous data, indicating that during EIU, the VEGF is also expressed in the ICB. It is noteworthy that topical LXA_4 treatment significantly reduces VEGF expression induced by LPS. Together, these data provide important evidence that lipoxins are capable of reducing the expression and antagonizing the effect of VEGF, suggesting these mediators as potent inhibitors of angiogenesis.

The signal transduction cascades elicited by LPS culminate in the activation of key transcriptional regulators, including NF-κB and AP-1. In most cell types, NF-κB complexes are found in the cytoplasm associated with IκBs, which prevent their nuclear localization. Cell stimulation and/or injury leads to the rapid degradation of IκB, allowing NF-κB translocation to the nucleus (Ghosh and Karin, 2002). Likewise, AP-1 is a transcriptional factor composed of the protein products of c-*jun* and c-*fos*, and their expression is stimulated by cytokines. Phosphorylation and activation of c-Jun is catalyzed by the mitogen-activated protein kinase family members (Angel and Karin, 1991). The activation of NF-κB and AP-1 is associated with the increased transcription of a number of genes involved in immune responses, including those encoding TNF-α, IL-1β, COX-2, and VEGF. It is interesting that recent evidence indicates that lipoxins and its analogs prevent the activation of both transcriptional factors, reducing inflammatory protein expression. In fact, it has been demonstrated that IL-1β-induced AP-1 and NF-κB DNA binding complexes are down-regulated by LXA_4 in human synovial fibroblasts (Sodin-Semrl et al., 2000, 2004). Furthermore, LXA_4 may affect the inflammatory process by suppressing the activation of NF-κB and AP-1 in human leukocytes (József et al., 2002). Consistent with previous reports, we detected p65 NF-κB and c-Jun activation in response to EIU. Our data also indicate that topical LXA_4 treatment reduces the migration of p65 NF-κB and c-Jun from the cytoplasm to the nucleus in the ICB and iris tissue, suggesting a reduction in the NF-κB and AP-1 transcriptional activity. These results confirm and extend those of previous studies showing that LXA_4 constitutes an important modulator of inflammatory gene expression.

Overall, our study demonstrates that topical LXA_4 treatment results in a reduction of the inflammatory process in EIU. A possible mechanism for this effect of LXA_4 seems to be associated with its ability to activate a signaling cascade that results in the repression of NF-κB and AP-1 activation and of proinflammatory mediator's production. Together, the present findings suggest that LXA_4 stable analogs might constitute attractive and new therapeutic alternatives to currently available anti-inflammatory drugs for the management of ocular inflammatory diseases.

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