

Amelioration of Experimental Autoimmune Uveoretinitis by Aldose Reductase Inhibition in Lewis Rats

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PURPOSE. Recently, the authors showed that the inhibition of aldose reductase (AR) prevents bacterial endotoxin-induced uveitis in rats. They have now investigated the efficacy of AR inhibitors in the prevention of experimental autoimmune-induced uveitis (EAU) in rats.

METHODS. Lewis rats were immunized with bovine interphotoreceptor retinoid-binding peptide (IRBP) to develop EAU. Two or 8 days after immunization, the rats started receiving the AR inhibitor fidarestat (7 mg/kg/d; intraperitoneally). They were killed when the disease was at its peak; aqueous humor (AqH) was collected from one eye, and the other eye of each rat was used for histologic studies. The protein concentration and the levels of inflammatory markers were determined in AqH. Immunohistochemical analysis of eye sections was performed to determine the expression of inflammatory markers. The effect of AR inhibition on immune response was investigated in isolated T lymphocytes.

RESULTS. Immunization of rats by IRBP peptide resulted in a significant infiltration of leukocytes in the posterior and the anterior chambers of the eye. Further, EAU caused an increase in the concentration of proteins, inflammatory cytokines, and chemokines in AqH, and the expression of inflammatory markers such as inducible-nitric oxide synthase and cyclooxygenase-2 in the rat eye ciliary bodies and retina. Treatment with fidarestat significantly prevented the EAU-induced ocular inflammatory changes. AR inhibition also prevented the proliferation of spleen-derived T cells isolated from EAU rats in response to the IRBP antigen.

CONCLUSIONS. These results suggest that AR could be a novel mediator of bovine IRBP-induced uveitis in rats. (*Invest Ophthalmol Vis Sci.* 2011;52:8033–8041) DOI:10.1167/iovs.11-7485

Uveitis, an inflammation of the middle vascular layer of the eye, is one of the most common causes of blindness and visual impairment worldwide. The annual incidence rate of uveitis varies between 17 and 52 cases per 100,000,^{1–4} and its prevalence varies between 69 and 204 cases per 100,000 population.^{1,2,5,6} In the Western countries uveitis is estimated to account for approximately 10% of the visual handicap; ~35%

of all uveitis patients have been reported to have significant visual impairment or legal blindness.^{7,8} Human autoimmune uveoretinitis includes an array of ocular inflammatory diseases such as sarcoidosis, sympathetic ophthalmia, birdshot retinochoroidopathy, Vogt-Koyanagi-Harada's disease, and Behçet's disease, which are often caused either by an autoimmune response or an unknown etiology.^{9–13} Immunization of rodents with interphotoreceptor retinoid-binding peptide 1169 to 1191 (IRBP) to induce experimental autoimmune uveoretinitis (EAU) is a widely used experimental model to investigate the pathophysiology of uveitis and to search for novel and effective therapeutic agents.¹⁴

EAU is a CD4⁺ T-helper cell type 1 (Th-1)-dominant autoimmune disease¹⁵ that involves the activation of various redox-sensitive signaling intermediates, including the transcription factor nuclear factor- κ B (NF- κ B).^{16,17} NF- κ B is activated by myriad stimulants, including cytokines, chemokines, and growth factors, by the generation of reactive oxygen species (ROS).^{17–19} NF- κ B transcribes various genes encoding proinflammatory cytokines, chemokines, cell surface receptors, adhesion molecules, and other inflammatory enzymes such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in innate and adaptive immune responsive cells, resulting in the cellular migration and infiltration of leukocytes in the ocular tissues. These proinflammatory mediators subsequently perpetuate the disease in an autocrine and paracrine fashion by further activating redox-sensitive signaling molecules. Therefore, the regulation of NF- κ B activity could be beneficial in controlling the inflammation. We have previously shown that inhibitors of aldose reductase (AR) successfully prevented the acute form of ocular inflammation induced by the bacterial endotoxin LPS in rats by blocking the activation of NF- κ B and inhibiting the release of inflammatory cytokines such as TNF- α and the inflammatory markers prostaglandin E2 (PGE2) and nitric oxide (NO).²⁰ A number of evidence suggests that the pathogenesis of EAU also involves the role of oxidative stress-mediated activation of molecular signals.^{21–23} Given that our previous results suggest that AR mediates oxidative stress signals in an infection-induced uveitis model in rats²⁰ and that AR inhibition prevented the activation of redox-sensitive transcription factors, we investigated the effect of AR inhibition on the pathogenesis of autoimmune-induced uveitis in rats by using a highly specific and a potent AR inhibitor, fidarestat. This inhibitor has been found to be safe and well tolerated in a 52-week clinical trial for diabetic neuropathy.²⁴

Our results suggest that the treatment of rats with an AR inhibitor significantly prevented leukocyte infiltration and increased protein concentrations, inflammatory cytokines and chemokines in rat AqH, and expression of inflammatory marker proteins such as COX-2 and iNOS in the rat retina. Further, the increased proliferation of spleen-derived T lymphocytes and the release of IL-17 by T cells from EAU rats in response to IRBP antigen were significantly prevented by AR

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inhibition. These results indicate an important role of AR in the pathogenesis of EAU, which warrants detailed investigation.

MATERIALS AND METHODS

Materials

The IRBP-derived peptides were synthesized and purified by CHI-Scientific (Maynard, MA). The peptide sequence was derived from bovine IRBP, peptide 1169-1191 (PTARSVGAADGSSWEGVVPDV). Complete Freund's adjuvant (CFA) was purchased from Sigma-Aldrich (St. Louis, MO). RPMI-1640 medium, phosphate-buffered saline (PBS), gentamicin sulfate solution, penicillin and streptomycin, trypsin/EDTA solution, and fetal bovine serum were purchased from GIBCO BRL Life Technologies (Grand Island, NY). Fidarestat was obtained as a gift chemical from Sanwa Kagaku Kenkyusho Co. Ltd. (Nagoya, Japan). Dimethyl sulfoxide (DMSO) was obtained from Fischer Scientific (Pittsburg, PA). Antibodies against iNOS, COX-2, and ICAM-1 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and CD68 antibodies were purchased from Abcam (Cambridge, MA). Membrane-based cytokine array system was purchased from RayBiotech, Inc. (Norcross, GA). All other reagents used were of analytical grade.

Animals

Male Lewis rats, 8 weeks old, were obtained from Harlan Laboratories (Indianapolis, IN) and were maintained in a pathogen-free condition in the animal resource center at the University of Texas Medical Branch in Galveston, Texas, where food and water were provided ad-libitum. The animals were housed under 12-hour light/12-hour dark cycles. All studies were conducted in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Induction of EAU

The rats were immunized by subcutaneous injections into two hind thighs with 100 μ g bovine IRBP in 100 μ L emulsion with CFA (1:1, vol/vol) supplemented with 2.5 mg/mL heat-killed *Mycobacterium tuberculosis* H37Ra (Difco Laboratories, Detroit, MI). Concurrently, an intraperitoneal injection containing 0.5 μ g purified *Bordetella pertussis* toxin (PTX; Calbiochem, San Diego, CA) in 100 μ L PBS was administered as an additional adjuvant.

AR Inhibitor Treatment

From the second day onward, the rats were injected (intraperitoneally) with the AR inhibitor fidarestat (7 mg/kg/d) in a volume of 0.05 mL DMSO daily. In experiments in which ARI was given therapeutically, fidarestat was administered on the day of onset of EAU (day 8). Control rats received equivalent amounts of the carrier (DMSO) alone.

Pathologic Assessment of EAU

The severity of EAU and inflammation was scored (range, 0 to 4) by an expert ophthalmologist blinded to the experimental groups who used a slit lamp microscope starting on the day of immunization (day 1) and continuing every other day until the end of the experiment. Grade 0 represented no disease (eyes reflected light [red reflex] and were translucent); grade 1 represented enlargement of the iris vessel and abnormal pupil contraction; grade 2 represented cellular infiltrates and hazy anterior chamber with decreased red reflex; grade 3 represented moderately opaque anterior chambers, still visible pupils, and dull red reflex; and grade 4 represented opaque anterior chambers, obscured pupils, and absence of red reflex.²⁵

Histology

The animals were killed during peak disease activity (day 14 or 18), and aqueous humor (AqH) was harvested immediately from one eye by an anterior chamber puncture using a 30-gauge needle under the surgical microscope. Total protein concentration in the AqH samples was measured using a Bio-Rad (Hercules, CA) protein assay kit. Aqueous humor samples were stored in ice water until use.

The eyes were enucleated and immediately stored in special fixation solution for 24 hours at 4°C. They were then transferred in 70% alcohol and stored at 4°C until embedded in paraffin. Sagittal sections (5 μ m) were cut and stained with hematoxylin and eosin. For histopathologic evaluation, the iris-ciliary body complex, anterior chamber, vitreous, and retina were observed under a light microscope.

Determination of PGE2 and NO Levels in Aqueous Humor

PGE2 and NO levels in the AqH were assessed with a commercially available rat cytokine antibody array system, in accordance with the manufacturer's (R&D Systems, Minneapolis, MN) instructions. AqH

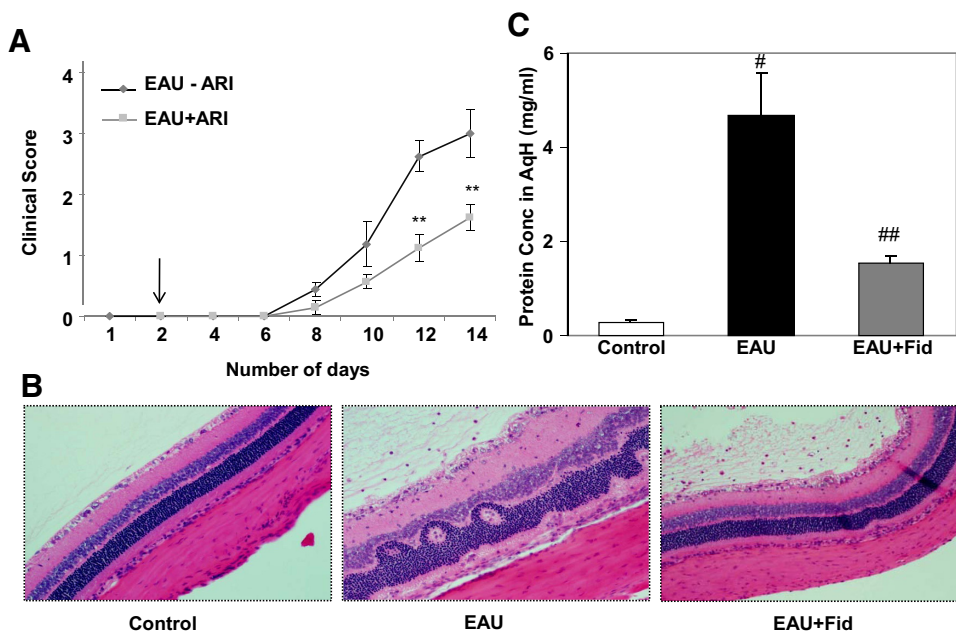


FIGURE 1. AR inhibition prevents IRBP-induced pathologic changes in the retina of the Lewis rat. **(A)** Clinical scores of EAU- and fidarestat-treated EAU eyes are shown through the disease course starting on day 2 after immunization. Data presented are the average of four to six rats per group. $^{**}P < 0.01$ versus EAU. (arrow) Start of fidarestat administration. **(B)** Representative photomicrograph of rat eye histopathology from each group is shown ($n = 4-6$). Magnification, 200 \times . **(C)** Total protein concentration in the AqH was measured by the Bradford method. Results are expressed as the mean \pm SD ($n = 8-12$). $^{\#}P < 0.001$ vs. Control. $^{##}P < 0.01$ vs. EIU group.

from at least four rats was pooled and diluted using assay buffer. Bars represent the percentage increase over control ($n = 4$).

Immunohistochemical Studies

Paraffin sections were warmed at 60°C for 1 hour and deparaffinized in xylene; this was followed by rehydration by passing through 100%, 95%, 80%, and 70% ethanol and finally in deionized water. After peroxidase blocking with 3% H₂O₂, the sections were rinsed in PBS twice for 5 minutes each and were incubated with blocking buffer (2% BSA, 0.1% Triton X-100, 2% normal rabbit IgG, and 2% normal goat serum) overnight at 4°C. Sections were incubated with antibodies against iNOS (1:100 dilution), COX-2 (1:100 dilution), ICAM (1:400 dilution), and CD68 (1:400 dilution) for 1 hour at room temperature. They were then incubated with a peroxidase-based visualization kit (Universal LSAB+ System-HRP; DakoCytomation, Carpinteria, CA) or FITC-labeled (for ICAM) and Texas red-labeled (for CD68) secondary antibodies. Slides were mounted with mounting medium (Vectashield; Vector Laboratories, Burlingame, CA) without or with DAPI. Sections were examined under bright-field light or fluorescent (using FITC and Texas red filters) microscopy (EPI-800; Nikon, Tokyo, Japan) and photographed with Nikon camera fitted to the microscope.

T-Cell Proliferation Assay Using MTS Assay

The T-cell proliferation assay was performed with spleen-derived T lymphocytes. T cell-enriched fractions were prepared by passing the dispersed cells from the spleen over nylon-wool columns. Nylon wool nonadherent cells (2×10^5 /well) were cultured with irradiated syngeneic spleen cells as antigen-presenting cells in quadruplicate and incubated with various concentrations of peptide in a 96-well flat-bottom microtiter plate for 72 hours at 37°C in the absence or presence of fidarestat. The MTS dye (Promega, Madison, WI) was added to the wells and incubated for 3 hours. At the end of incubation, the plates were read at 490 nm using a multiwell ELISA plate reader. Absorbance at 490 nm represented T-cell growth in response to antigen; the data are presented as mean \pm SD absorbance.

Determination of Inflammatory Markers

The levels of cytokines and chemokines in the AqH were determined with a commercially available rat-specific cytokine antibody array system according to the manufacturer's (Ray Biotech, Norcross, GA) instructions. Densitometry analysis of the array was performed with an imaging system (Image Station 440CF; Eastman Kodak, Rochester, NY). IL-17 cytokine produced in the T-cell culture supernatant was quantified to assess the antigen-specific immune response in vitro. Spleen-derived T cells (5×10^5 /well) were cultured with IRBP without or with fidarestat for 72 hours. At the end of incubation, culture media were harvested, cleared by centrifugation (5000 rpm; 5 minutes), and used for the IL-17 determinations using a rat cytokine antibody array system (Cosmo Bio USA, Carlsbad, CA) according to the manufacturer's instructions.

Statistical Analysis

Data are expressed as the mean \pm SD of 8 to 12 animals per group. Experiments were carried out twice with four to six animals for each treatment. All the data were analyzed by Student's *t*-test using data analysis software (Excel 2003; Microsoft, Redmond, WA). $P < 0.05$ was considered statistically significant.

RESULTS

AR Inhibition Ameliorates EAU in Lewis Rats

First, we examined whether inhibition of AR by fidarestat suppresses ocular inflammation in EAU rats. Rats were examined every other day following immunization for clinical signs of EAU by slit lamp microscope. On day 14, when the disease was at peak, the mean histopathologic score of EAU was $2.7 \pm$

0.51 in the IRBP-immunized rats, which was significantly less in the rats treated with fidarestat (1.3 ± 0.5 ; $P < 0.01$) (Fig. 1A). Histopathologic examination of the retinas of control rats showed normal stratiform morphology with all the layers intact; however, in EAU rats, the retina was detached, the photoreceptor layer was markedly damaged with severe edema at numerous loci, and there was severe infiltration of leukocytes in the posterior and anterior chambers. The fidarestat-treated rats showed significantly milder pathologic severity of retina than did the EAU rats (Fig. 1B), and, although leukocytes had infiltrated the posterior and anterior chambers, their numbers were markedly ($\sim 50\%$) reduced. Further, the protein concentration in the AqH of EAU rats was significantly (~ 17 -fold; $P < 0.01$) higher than in the AqH of control rats, which was significantly ($\sim 70\%$) prevented in fidarestat-treated rats (Fig. 1C). Similar results were observed when fidarestat was given therapeutically after onset of the disease (Figs. 2A, 2B).

AR Inhibition Prevents EAU-Induced PGE₂ and NO Levels in AqH

Next, we examined the levels of secreted inflammatory markers such as PGE₂ and NO in the AqH. As shown in Figure 3A, the mean level of PGE₂ increased by approximately 4-fold ($P < 0.005$) to 233 ± 86.6 pg/mL in the EAU group, which was 61 ± 2.1 pg/mL in controls. In the IRBP-immunized and fidarestat-treated rats, there was a significantly decreased (78%) level of

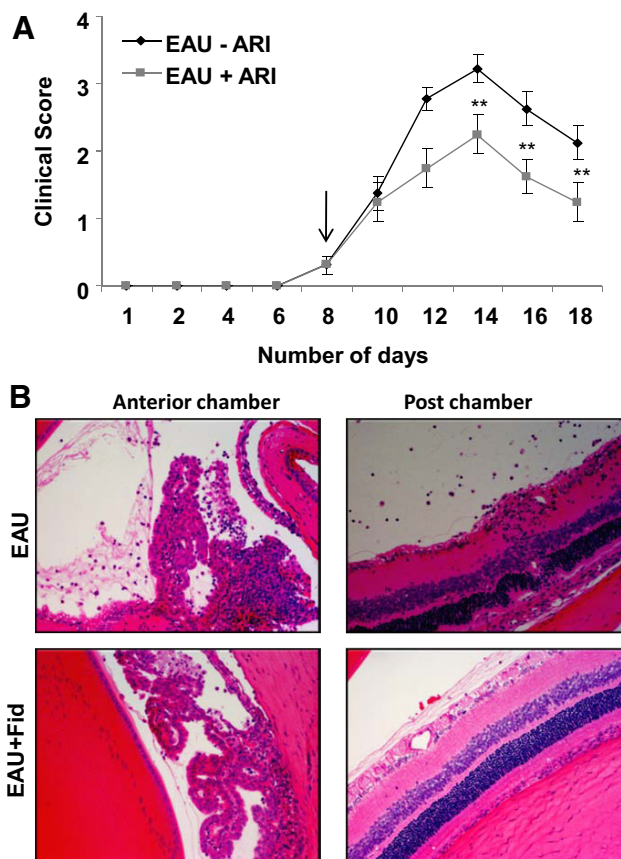


FIGURE 2. AR inhibition ameliorates IRBP-induced pathologic changes in the rat eyes. (A) Clinical scores based on examination of rat eyes examined through the disease course starting on the day of immunization are shown. Data represent mean \pm SD ($n = 5$). $**P < 0.01$ vs. EAU group. Arrow: start of fidarestat administration. (B) Representative photomicrograph (both anterior and posterior chambers) showing histopathology of the rat eyes from EAU- and fidarestat-treated EAU is shown ($n = 5$). Magnification, 200 \times .

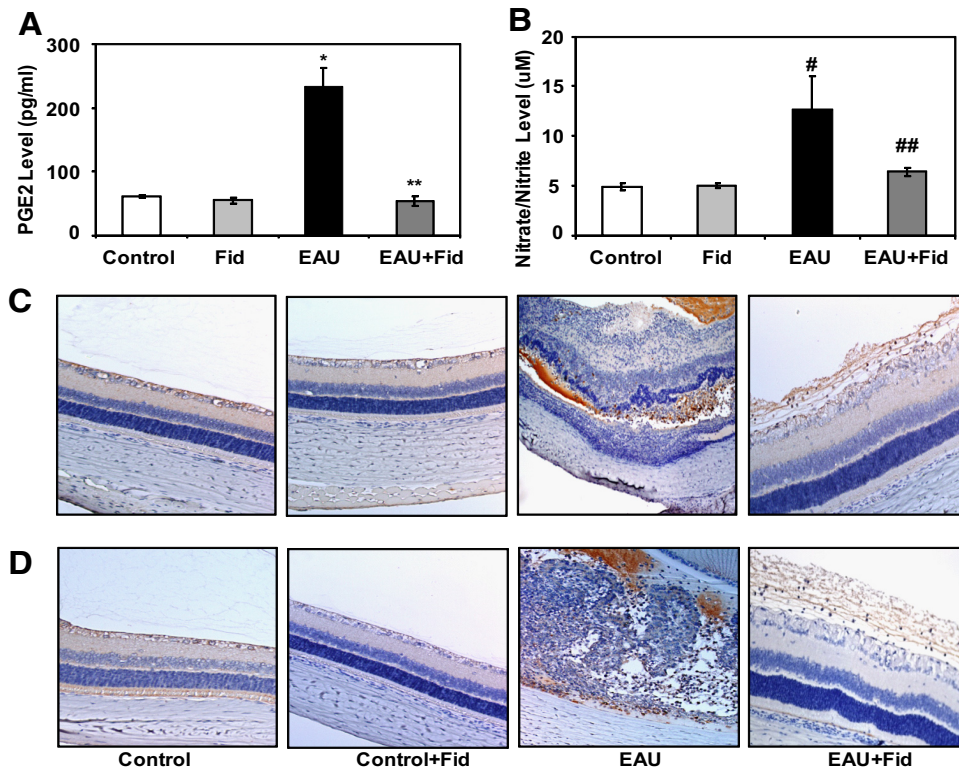


FIGURE 3. Inhibitory effect of AR inhibitor on the inflammatory markers in the AqH of EAU rats after 14 days of immunization. The levels of (A) PGE2 and (B) NO were determined using specific commercial kits. Results are expressed as mean \pm SD ($n = 4$). * $P < 0.005$ vs. Control. ** $P < 0.002$ vs. EAU. # $P < 0.02$ vs. Control. ## $P < 0.05$ vs. EAU. The eye sections were stained with antibodies against (C) COX-2 and (D) iNOS. Magnification, 200 \times .

PGE2 in AqH ($P < 0.003$ vs. EAU). Similarly, the NO level in the AqH of EAU rats increased by more than 2-fold compared with control ($P < 0.03$), and treatment of EAU rats with fidarestat decreased the NO levels in AqH by approximately 70% ($P < 0.05$) (Fig. 3B).

Next we examined the expression of the enzymes that catalyze the production of PGE2 and NO (COX-2 and iNOS, respectively) by immunohistochemistry. As shown in Figures 3C, 3D there was increased COX-2- and iNOS-specific staining in the EAU eye sections, especially in the retina and where infiltrated cells were accumulated in the posterior segment; such changes were not observed in the AR inhibitor-treated rat eyes. Taken together, these observations suggest that increased PGE2 and NO levels in AqH and expression of COX-2 and iNOS in the retina are associated with EAU-related inflammation; treatment with AR inhibitor significantly blocked the inflammatory changes.

AR Inhibition Prevents the EAU-Induced Increase of Inflammatory Cytokines and Chemokines in AqH

Increases in a number of inflammatory cytokines and chemokines, especially of the Th-1 type, are known to be associated with autoimmune uveitis.^{26,27} We therefore determined the levels of inflammatory cytokines and chemokines in the AqH using membrane-based cytokine array. As shown in Figure 4, there was a significant increase in the secretion of cytokines (IL-1 α , IL-1 β , IL6, IL10, TNF- α , IFN- γ) and chemokines (GM-CSF, CINC2, CINC3, CNTF, LIX, MIP3 α , MCP-1) and of growth factors such as VEGF and β -NGF in the AqH of EAU rats. Treatment of EAU rats with fidarestat significantly prevented the release of cytokines, chemokines, and growth factors (Fig. 4). These results suggest that AR regulates the release of inflammatory markers in the eye, which is critical for the development of uveitis. Its inhibition could block the production of inflammatory markers.

AR Inhibition Suppresses Antigen-Induced T-Lymphocyte Proliferation and Immune Response in EAU Rats

Given that EAU is a T lymphocyte-mediated disease and it has been shown that T cells proliferate vigorously when exposed to immunized peptide,²⁸⁻³⁰ we next determined the efficacy of treatment with the AR inhibitor fidarestat on antigen-induced T-cell proliferation. Spleen-derived T cells from naive control rats, control rats treated with fidarestat, EAU rats, and EAU rats treated with fidarestat were isolated using nylon wool column and were subsequently incubated without or with peptide antigen (IRBP; 25 μ g/mL) in the presence and absence of fidarestat for 72 hours, and their proliferation was measured by MTS assay. As shown in Figure 5, T cells from the control and the control + fidarestat groups did not show any increase in proliferation (OD, 0.23 ± 0.01 and 0.216 ± 0.004 , respectively) when stimulated with IRBP. Conversely, though proliferation occurred even in the absence of peptide, those from the EAU group showed a significant increase in proliferation (OD, 0.57 ± 0.08 ; $P < 0.02$) when stimulated with IRBP. On treatment with fidarestat, IRBP-induced proliferation of EAU-derived T cells decreased significantly (OD, 0.29 ± 0.04 ; $P < 0.04$). Further, the induction of T lymphocytes from the EAU + fidarestat group showed mild proliferation in the presence of the peptide, which was significantly (OD, 0.35 ± 0.47 ; $P < 0.05$) lower than in IRBP-challenged, EAU-derived T cells, and almost no proliferation was observed when fidarestat was added with IRBP (OD, 0.21 ± 0.003). Antigen-specific T-cell proliferation in the in vivo ARI-treated group was significantly ($P < 0.01$) lower than in nontreated rats (first column in EAU and EAU + fidarestat groups). These results suggest that AR inhibition significantly blocked the antigen-induced proliferative response of the T cells and thereby could prevent the inflammatory response.

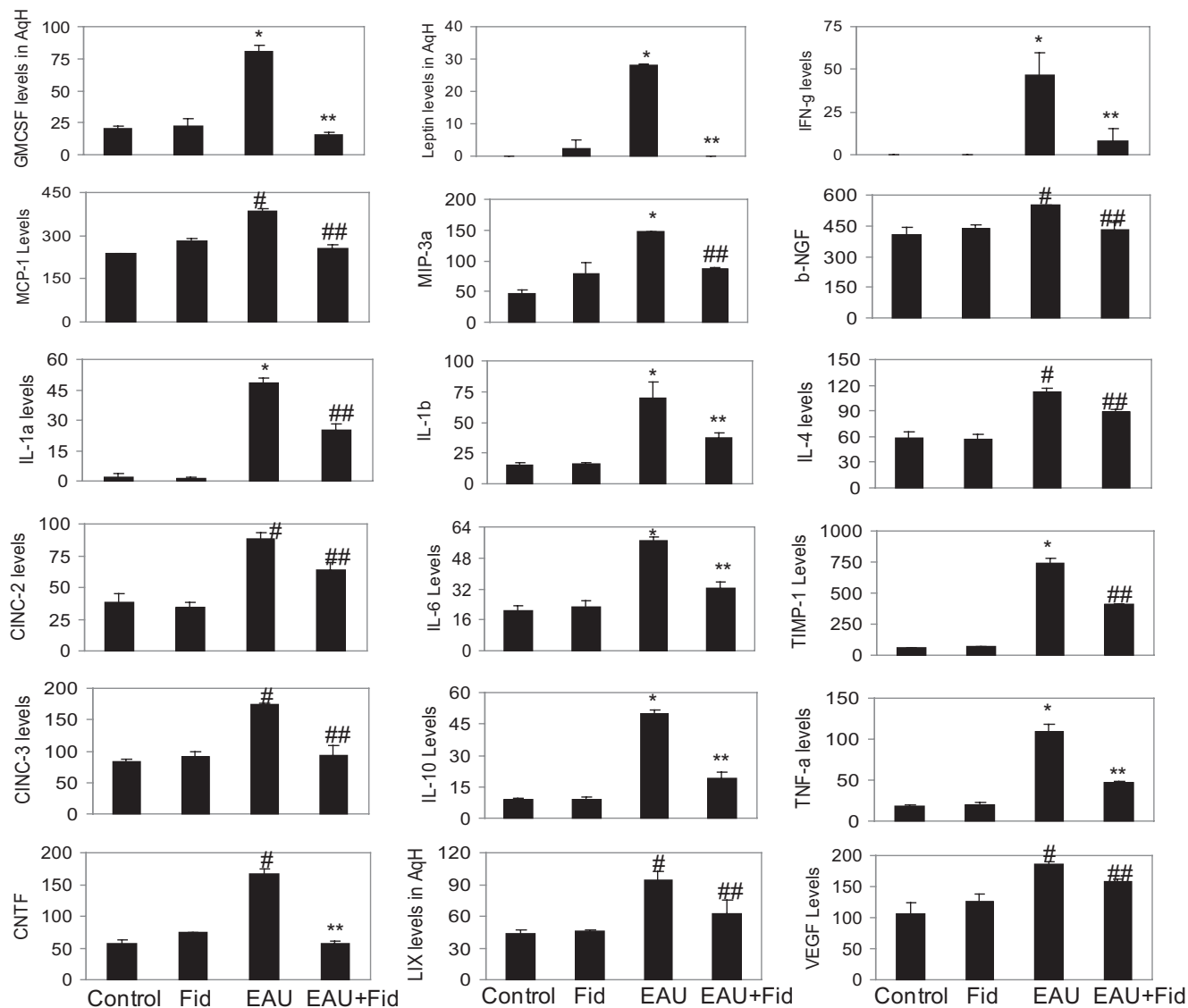


FIGURE 4. AR inhibition reduces cytokine and chemokine concentrations in the AqH of EAU rats. The expression of various cytokines, chemokines, and growth factors was determined using membrane-based ELISA ($n = 4$). The values represent arbitrary units of pixel density for cytokine-specific spots on the array determined by densitometry. * $P < 0.001$ vs. Control. ** $P < 0.01$ vs. EAU. # $P < 0.02$ vs. Control. ## $P < 0.05$ vs. EAU.

AR Inhibition Suppresses Antigen-Induced Production of IL-17 by T Lymphocytes from EAU Rats

In addition to various other cytokines, IL-17, which is secreted by a subtype of T lymphocytes, the Th-17 cell, has been implicated in the pathogenesis of autoimmune uveitis.^{31,32} Therefore, we next determined the levels of IL-17 in the T-cell culture media after activation with the antigen. As shown in Figure 6, there was increased secretion of IL-17 in the culture medium by spleen-derived T lymphocytes from EAU rats, even in the absence of IRBP, that increased further in response to antigen stimulation; treatment of the cells with fidarestat significantly prevented the release of IL-17. These results thus suggest that IL-17 is released by activated T cells in their surroundings, which could play a significant role in the pathogenesis of uveitis. They also suggest that AR regulates the T cell-mediated immune response in the eye, which is critical for the development of uveitis, and that inhibition by the AR

inhibitor blocks the production of inflammatory cytokines that help in disease progression.

AR Inhibition Suppresses the Induction of Adhesion Molecules on Retinal Endothelium in EAU Rats

Adhesion molecules such as ICAM-1 on vessel walls help the migration of leukocytes to the injury site.³³ Therefore, we examined the expression of ICAM-1 in the retinal vessels in EAU rats. Increased expression of ICAM-1 was observed in the EAU retina compared with controls, and treatment with fidarestat significantly prevented these changes (Fig. 7). A significant increase in the expression of CD68, a macrophage marker, which colocalized with the blood capillaries, was also observed, suggesting the activation of macrophages and their migration to the inflammation site in retina. These results collectively suggest that AR inhibition could prevent the expression of adhesion markers and thus could block the migra-

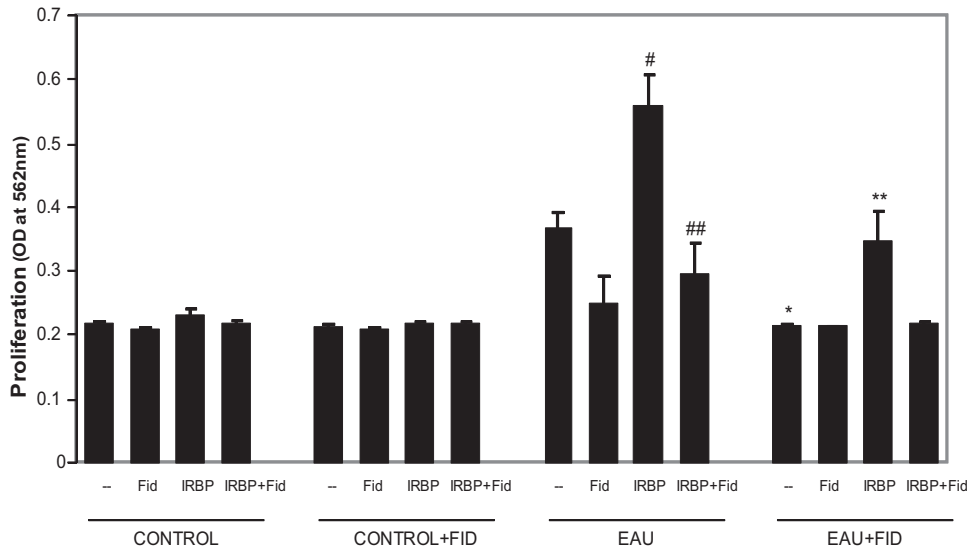


FIGURE 5. Inhibitory effect of the AR inhibitor fidarestat on the proliferation of spleen-derived T cells after stimulation with IRBP. The proliferation of cultured cells was examined by MTS assay. The results are expressed as mean \pm SD ($n = 4$). # $P < 0.02$ vs. Control cells treated with IRBP alone. ## $P < 0.04$ vs. EAU cells treated with IRBP alone. * $P < 0.01$ vs. untreated EAU-derived T cells. ** $P < 0.05$ vs. EAU cells treated with IRBP alone.

tion of inflammatory cells, thereby preventing the pathogenesis of EAU in rats.

DISCUSSION

Human indigenous uveitis has marked similarity with the EAU in experimental animals because both involve the activation of immune cells, including macrophages and T cells, with Th-1 polarization.^{14,34,35} The activated immune cells secrete various cytokines, chemokines, and inflammatory mediators, resulting in severe ocular inflammation and tissue damage. Several investigators have implicated the role of reactive oxygen species (ROS) in disease initiation and progress.^{20,22,36} ROS are well known to activate redox-sensitive signaling intermediates that result in the transcription of several inflammatory mediators that participate in the induction of various pathologic conditions, including uveitis.^{20,37,38} Therefore, regulation of the ROS-induced molecular signals could be a better therapeutic approach for autoimmune uveitis in humans. Indeed, antioxidants have been suggested for the amelioration of ocular inflammation in autoimmune uveitis.^{16,39}

We have recently demonstrated that inhibition of AR significantly prevented endotoxin-induced uveitis in rats by blocking the ROS-induced signaling that activates NF- κ B.²⁰ However, the pathogenesis of EAU is different from that of EIU because

EAU is a T cell-mediated disease that primarily involves the production of Th-1 and recently implicated Th-17 cytokines by infiltrating T lymphocytes.¹⁴ Moreover, in EAU, the infiltration of phagocytic cells in the outer retinal layer, including the photoreceptor layer and uveal tract, occurs after the initial infiltration of mononuclear cells in the posterior segment of the eye, such as retinal perivascular space. In contrast, in EIU, leukocytes infiltrate the anterior segment of the eye, such as the ciliary body-iris complex and the aqueous chamber. Besides differences in the initiation and progression of pathogenesis, there are several underlying similarities between these ocular diseases because oxidative stress is common denominator in both.^{21,40-42} Further, it has been observed that in both the cases inflammation is regulated by redox-sensitive molecular signals.^{16,17,20,37} We have previously demonstrated that the inhibition of AR ameliorated EIU in rats,²⁰ and we envisaged that blocking the ROS signals and subsequent activation of the signaling cascade that activates redox-sensitive transcription factors NF- κ B and AP-1 should also be beneficial in the prevention of autoimmune uveitis. Therefore, we investigated the efficacy of AR inhibition in EAU model in rats.

We observed that treatment of rats with the AR inhibitor fidarestat prevented the progression of the disease. On day 14, when the disease was at its peak pathologic score (approximately 3) in EAU rats, fidarestat-treated rats had a score of nearly 1.2, which was significantly lower ($P < 0.01$) than that in rats not treated with fidarestat. Further the retinal detachment, photoreceptor layer damage, edema, and severe infiltration of leukocytes in the posterior chamber and other pathologic markers of EAU were markedly prevented in the AR inhibitor-treated rats. Our results in the present study thus suggest that AR inhibitors could have therapeutic potential in chronic uveitis-related ocular inflammation (such as EAU in rats) in addition to its efficacy in acute ocular inflammation (i.e., in EIU rats). AR inhibition has been shown to be beneficial in other pathologic conditions involving acute (e.g., sepsis, allergic asthma) and chronic (e.g., diabetic complication, tumorigenesis, metastasis, and neovascularization) inflammation.⁴³⁻⁵⁰ Given that EAU involves the activation of inflammatory mediators such as PGE2 and NO and AR is known to mediate it,^{51,52} we examined whether the inhibition of AR could suppress the levels of PGE2 and NO. Our results show that not only did AR inhibition prevent the release of these inflammatory mediators in the AqH, the expression of the enzymes that catalyze their production decreased in the retina.

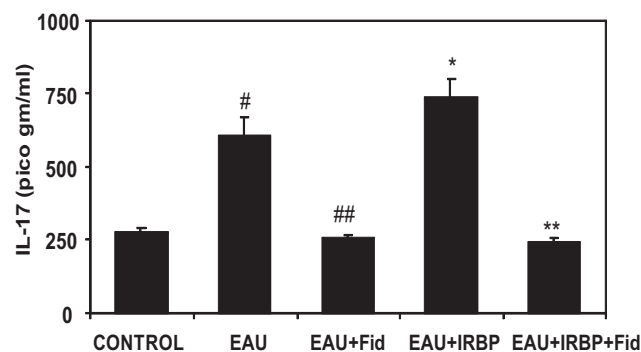


FIGURE 6. AR inhibition prevents increased IL-17 levels in the T-cell culture medium from EAU rats. IL-17 levels in the T-cell culture media were determined using a specific ELISA kit. Results are expressed as mean \pm SD ($n = 4$). # $P < 0.003$ vs. Control. ## $P < 0.005$ vs. EAU. * $P < 0.005$ vs. Control. ** $P < 0.002$ vs. EAU + IRBP.

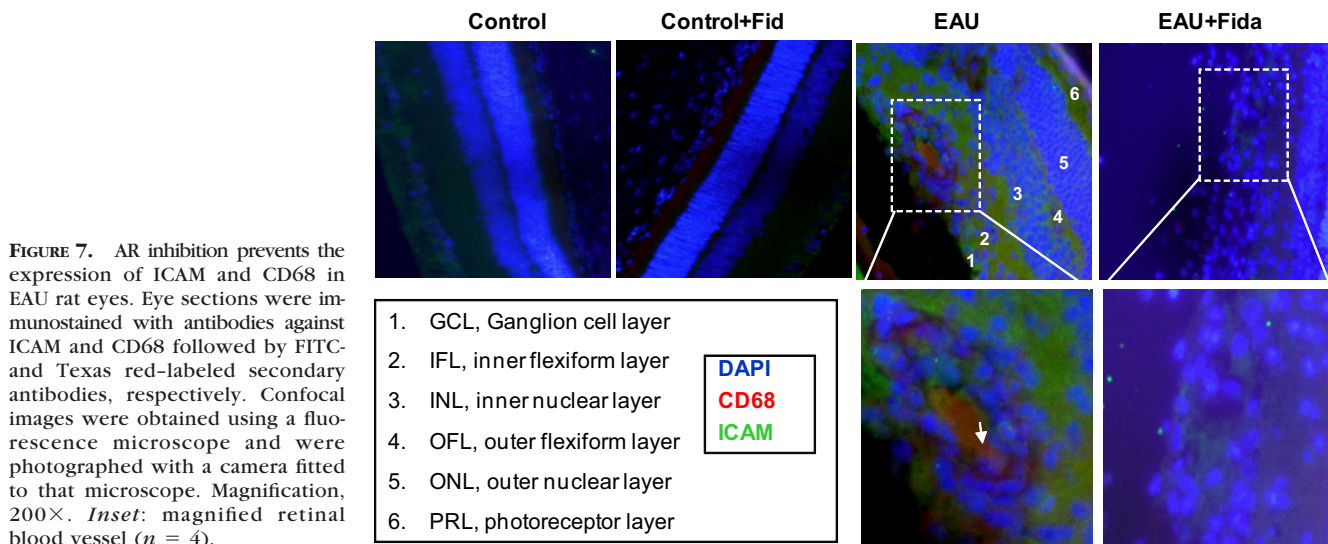


FIGURE 7. AR inhibition prevents the expression of ICAM and CD68 in EAU rat eyes. Eye sections were immunostained with antibodies against ICAM and CD68 followed by FITC- and Texas red-labeled secondary antibodies, respectively. Confocal images were obtained using a fluorescence microscope and were photographed with a camera fitted to that microscope. Magnification, 200 \times . *Inset:* magnified retinal blood vessel ($n = 4$).

The increased production of Th-1 cytokines and chemokines in the AqH was also prevented by AR inhibition, suggesting a probable prevention of polarized differentiation of naive T-helper cells into the Th-1 type by the AR inhibitor. Although the precise mechanism is still unclear, it is likely that inhibition of the proliferation of Th-1 cells could be the main contributor. We therefore isolated the T lymphocytes from the spleens of rats from control, control treated with ARI, EAU, and EAU treated with ARI groups and cultured them with IRBP without or with the AR inhibitor. Significantly increased proliferation of T cells from the spleens of EAU rats was observed that was prevented by AR inhibition. Similarly, T cells from AR inhibitor-treated EAU rats also showed slight proliferation in the presence of IRBP that was blocked by AR inhibitor treatment.

In addition to the involvement of Th-1-type lymphocytes in disease mediation, several recent studies have also implicated the Th-17 subset of T helper cells.^{51,53} To investigate the involvement of these cells, we examined the culture media of spleen-derived T cells cultured with IRBP without or with AR inhibitor for the presence of IL-17, a Th-17-specific cytokine. We observed that not only did T cells derived from EAU rats show increased levels of IL-17, when treated with antigen the IL-17 levels increased further, but AR inhibition prevented these changes, suggesting that there is distinct population of IL-17-secreting cells that increases when exposed to the uveitogenic antigen in vitro and that AR inhibition regulates their growth. Further, leukocyte migration to the inflammatory site is known to play a major role during autoimmune uveitis.⁵⁴ The activated leukocytes first loosely adhered to the endothelium of the blood vessel and then firmly adhered with the help of the expression of extracellular proteins such as CD68 on activated leukocytes and of adhesion molecules such as ICAM (CD54) on the endothelial cells. Then they transmigrated to the subendothelial space and further into the interstitial compartments. Although this event begins at the initiation of the disease, it also participates in the progression phase. Thus, preventing the expression of adhesion molecules and stopping the migration of inflammatory cells should be beneficial in uveitis.^{55,56} Immunofluorescence staining of the eye sections showed that the expression of ICAM on the endothelial and surrounding cells and of CD68 on leukocytes was prevented in AR inhibitor-treated rats, whereas in EAU rats there was significant fluorescence staining. Although our current studies indicate the AR inhibitor prevents EAU, further studies are needed to better determine treatment effects during the entire uveitis course. This is especially important because

the entire uveitis course, after AR inhibitor treatment, will provide more information such as whether the treatment delays the onset of disease and reduces the disease course or, on the other hand, whether it could delay the onset of disease and disease resolution.

How the inhibition of AR prevents disease development is not yet clear. Nevertheless, from the evidence we have observed in our other studies,⁵⁷⁻⁵⁹ it can be stated that oxidative stress-induced peroxidation of membrane lipids generates lipid-derived aldehydes that, along with their glutathione conjugates, are reduced by AR to their respective lipid alcohols, which in turn have been shown to activate redox signaling, cause inflammation, and add to the already prevailing oxidative stress. This starts a cycle of events that leads to disease establishment and progression. By inhibiting AR, the formation of glutathione-lipid alcohols is blocked, which would break the vicious circle of oxidative stress-induced inflammation and prevent disease progression.

Several AR inhibitors, such as sorbinil, tolrestat, and zenarstat, have been tested in clinical trials for diabetic complications but nonspecificity, and the requirement of higher doses led to the failure of their development in clinically safe drugs.⁶⁰ However, these efforts led to the synthesis of a highly specific and potent AR inhibitor, fidarestat, which has already been used in diabetic neuropathy patients and has been found to be safe and well tolerated.²⁴ Similarly, long-term treatment with epalrestat at the dose of 150 mg/d was well tolerated.⁶¹ These studies present encouraging evidence for the use of AR inhibitors for therapeutic intervention in inflammatory diseases other than diabetic complications such as uveitis.

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