Immunology and Microbiology

Inhibitory Effect of Aminoimidazole Carboxamide Ribonucleotide (AICAR) on Endotoxin-Induced Uveitis in Rats

Jun Suzuki, Akrivi Manola, Yusuke Murakami, Yuki Morizane, Kimio Takeuchi, Maki Kayama, Joan W. Miller, Lucia Sobrin, and Demetrios G. Vavvas

PURPOSE. To investigate the anti-inflammatory effect of amino-imidazole carboxamide ribonucleotide (AICAR), an analog of adenosine monophosphate (AMP), in endotoxin-induced uveitis (EIU).

METHODS. EIU was induced by subcutaneous injection of lipopolysaccharide (LPS) (200 μg) in Lewis rats. AICAR (50 mg/kg, intraperitoneally) was given 6 hours prior and at the same time as LPS injection. Clinical uveitis scores, number of anterior chamber (AC) infiltrating cells, anterior chamber protein concentration, retinal vessel leukocyte adhesion, and protein leakage were measured 24 hours later. Protein levels of C-C chemokine ligand-2 (CCL-2)/monocyte chemotactic protein-1 (MCP-1), tumor necrosis factor-α (TNF-α) and intercellular adhesion molecule-1 (ICAM-1) in aqueous humor and retina and nuclear translocation of nuclear factor–κB (NF-κB) in the retina were determined by enzyme-linked immunosorbent assay (ELISA). Both mRNA and protein levels of CD14 in peripheral blood mononuclear cells were also measured.

RESULTS. AICAR treatment significantly reduced EIU clinical severity as well as inflammatory cell infiltration and protein concentration in aqueous humor. Similarly, the number of retinal vessel-adherent leukocytes and protein leakage were decreased by AICAR treatment. Protein levels of TNF-α, CCL-2/MCP-1, and ICAM-1 in aqueous humor and CCL-2/MCP-1 and ICAM-1 levels in retina were suppressed with AICAR treatment. AICAR also reduced NF-κB translocation and CD14 expression.

CONCLUSIONS. AICAR reduces systemic LPS susceptibility and attenuates intraocular inflammation in a rat EIU model by limiting infiltration of leukocytes, suppressing inflammatory mediators, and inhibiting the NF-κB pathway. (*Invest Ophthalmol Vis Sci.* 2011;52:6565–6571) DOI:10.1167/iovs.11-7331

Uveitis, an inflammatory eye disorder of the uveal tract and contiguous structures, is a significant cause of visual loss.[1,2] In some cases of human uveitis, immunosuppressive drugs including corticosteroids, antimetabolites and biologics are needed to control the inflammatory process. However, there are patients who do not respond or cannot tolerate these agents.[3,4] Therefore, other treatment options in uveitis are needed.

Endotoxin-induced uveitis (EIU) is a rodent model of human uveitis, induced by a single systemic injection of endotoxin or lipopolysaccharide (LPS).[5] These animals develop acute bilateral anterior inflammation, characterized by a breakdown of the blood–ocular barrier and accumulation of inflammatory cells. Various mediators such as interleukin (IL)-6, tumor necrosis factor-α (TNF-α), C-C chemokine ligand-2 (CCL-2)/monocyte chemotactic protein-1 (MCP-1) contribute to the development of EIU.[6-8] Inflammation in the posterior segment of the eye is seen in EIU with the adherence of recruited leukocytes to the retinal vascular endothelium, by expression of intercellular adhesion molecule-1 (ICAM-1) and the breakdown of the blood–retinal barrier.[7,9,10] EIU has been used to investigate the pathogenesis of uveitis and to evaluate the therapeutic effect of several drugs.[11-14] CD14 is coreceptor for LPS and expressed mainly on monocytes, macrophages, and neutrophils and its association with Toll-like receptor 4 leads to activation of transcriptional factors, including nuclear factor–κB (NF-κB).[15] There have been reports that suppression of CD14 expression has a potent therapeutic effect on reducing LPS-induced inflammation[16] including EIU.[17]

AMP-activated protein kinase (AMPK) is a metabolic stress-sensing enzyme that allows a cell to modify cellular pathways to reduce energy usage and increase energy production.[18-20] A cell-permeable activator of AMPK, 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside (AICAR), has been examined in animal models of metabolic diseases.[21] The therapeutic effect of AICAR has been reported in disease states including ischemia and reperfusion heart injury,[22] diabetes,[23] and B-cell chronic lymphocytic leukemia.[24] Recently, AICAR has been shown to have an anti-inflammatory effect through inhibition of LPS-induced proinflammatory mediators including TNF-α, IL-1β, and IL-6.[25] It has also been reported to reduce the inflammation in acute lung injury[26] and autoimmune diseases such as neuritis via AMPK activation.[27,28] In vitro studies have shown AICAR's possible therapeutic potential for treating retinoblastoma cell proliferation[29] and the inflammatory response of retinal pigment epithelium (RPE) cells.[30] However, its ability to suppress uveitis-related intraocular inflammation has not yet been examined.

In the present study, we investigated whether AICAR can suppress ocular inflammation in EIU and then sought to understand the molecular mechanisms responsible for the anti-inflammatory effect.

METHODS

Endotoxin-Induced Uveitis and AICAR Treatment

All experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the

Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Care and Use Committee of the Massachusetts Eye and Ear Infirmary. Six groups of male Lewis rats (6–8 weeks old; Charles River, Wilmington, MA) were compared in these experiments: (1) normal: no treatment; (2) AICAR: normal rats treated with AICAR 6 hours before examination; (3) LPS: EIU rats treated with PBS; and (4) LPS with AICAR: EIU rats treated with AICAR. EIU induction was achieved with injection of 200 μg of LPS (*Salmonella typhimurium*; Sigma Chemical, St. Louis, MO) diluted in 0.1 mL PBS into one hind footpad. AICAR and PBS (for placebo treatment) were delivered via intraperitoneal injection of AICAR (50 mg/kg or 100 mg/kg body weight, Sigma) diluted in 150 μL PBS and equal volume of PBS, respectively, at 6 hours before and the same time as LPS injection. In addition to the pretreatment, we also examined the effect of AICAR (50 mg/kg), given only at same time as LPS injection (5), and 6 hours after LPS injection (6).

Clinical Evaluation of EIU

Clinical scoring of EIU was performed as previously described.[31] Slit lamp examination was conducted 24 hours after LPS injection in a masked fashion. The severity of EIU was graded from 0 to 4 by a masked investigator, using the scale: 0 = no inflammatory reaction; 1 = discrete inflammation of the iris and conjunctival vessels; 2 = dilation of the iris and conjunctival vessels with moderate flare in the anterior chamber; 3 = hyperemia in iris associated with Tyndall effect in the anterior chamber; and 4 = same clinical signs as 3 plus the presence of fibrin or synechiae.

Histopathologic Evaluation

EIU rats were euthanatized 24 hours after LPS injection. The eyes were enucleated immediately and stored in 10% formalin solution. They were then embedded in paraffin and 10-μm sagittal sections were cut and stained with hematoxylin and eosin. For histopathologic evaluation, the anterior and posterior chambers were examined with light microscopy.

Infiltrating Cells and Protein Concentration in Aqueous Humor

Aqueous humor was collected by anterior chamber puncture with a 30-gauge needle 24 hours after LPS injection. For cell counting, 1 μL of aqueous humor was diluted with PBS and suspended in an equal amount of Trypan-blue solution, and the cells were counted with a hematocytometer under a light microscope. A separate sample of aqueous humor was centrifuged at 2500 rpm for 5 minutes at 4°C and the total protein concentration was measured by Lowry methods with a protein assay kit (Bio-Rad, Hercules, CA). Aqueous samples were stored on ice until used; cell counts and total protein concentrations were measured on the day of sample collection.

Ex Vivo Evaluation of Retinal Vascular Adherent Leukocytes

Leukocyte adhesion to the retinal vessels was evaluated 24 hours after EIU induction by using the Concanavalin A (Con A) lectin staining technique.[32] After deep anesthesia, the chest cavity was opened and a 20-gauge perfusion cannula was introduced into the aorta. Rats were then perfused with 20 mL PBS to remove erythrocytes and nonadherent leukocytes, followed by 20 mL of fluorescein-isothiocyanate (FITC)-coupled Con A (Vector Laboratories, Burlington, CA) in PBS to stain the vascular endothelium and firmly adherent leukocytes. Subsequently, residual unbound Con A was removed with 20 mL PBS. The eyes were subsequently enucleated and the retinas were carefully flat-mounted. The flat-mounts were imaged using an epifluorescence microscope (DM RXA; Leica, Solms, Germany) and the total number of Con A-stained adherent leukocytes per retina was counted.

Evaluation for Retinal Vascular Leakage

Protein leakage from retinal vessels was assessed with a biotinylated bovine serum albumin (bBSA) assay as previously described.[10] At 23 hours after LPS injection, rats were anesthetized and 0.14 mL of 43.7 mg/mL bBSA (Santa Cruz Biotechnology, Santa Cruz, CA) was injected through the femoral vein. After 1 hour, the chest cavity was opened and the rat was perfused with lactated Ringer's solution via the left ventricle for 6 minutes. Subsequently, the eyes were enucleated and retinas were carefully removed and placed in cold PBS. The retinas were then placed in 500 μL of lysis buffer (Complete Protease Inhibitor Cocktail Tablets; Roche Diagnostics Corp., Indianapolis, IN) containing protease inhibitor, sonicated and centrifuged at 13,000 rpm for 10 minutes. Supernatant was collected and bBSA concentration was measured by enzyme-linked immunosorbent assay (ELISA). The degree of vascular leakage was estimated by measuring the protein concentration in each retina.

Preparation of Nuclear Extracts and Detection of NF-κB Activity

For the measurement of NF-κB activity, we used pooled retinas from six normal rats and nine EIU PBS-treated rats and nine EIU AICAR-treated rats. Retinas were homogenized and nuclear extracts were prepared (ProteoJet Cytoplasmic and Nuclear Protein Extraction Kit; Fermentas Inc., Burlington, Canada). The amount of NF-κB in the nuclear extracts was analyzed by measuring the p65 protein levels with a NF-κB detection kit (p65 Transcription Factor Assay kit; Active Motif, Carlsbad, CA). NF-κB concentration was calculated and corrected for protein concentration.

Detection of Cytokines and Chemokines in Serum, Aqueous Humor, and Retina

Under deep anesthesia, the animals were killed and the retinas were carefully removed 24 hours after LPS injection, placed into 200 μL of lysis buffer and then sonicated. The lysate was centrifuged at 13,000 rpm for 10 minutes at 4°C and supernatant was isolated. Three hours after LPS injection, blood samples were collected from the heart and subsequently centrifuged at 3000 rpm for 30 minutes at 4°C and serum samples were separated. Cytokine and chemokine levels in the serum, aqueous humor, and retina were assessed with the rat CCL-2/MCP-1 (Invitrogen, Camarillo, CA), rat TNF-α kit (R&D Systems, Minneapolis, MN) and rat ICAM-1 kit (R&D Systems).

Preparation of Peripheral Blood Mononuclear Cell (PBMC) Samples

Three hours after LPS injection, blood samples were collected and PBMC samples were isolated by density gradient centrifugation (Histopaque 1083; Sigma). The red blood cells were lysed (Red Blood Cell Lysing Buffer; Sigma).

Western Blot Analyses for CD14

Pooled PBMCs from three rats were homogenized with lysis buffer (Roche Diagnostics Corp.) and centrifuged at 13,000 rpm for 10 minutes at 4°C. Thirty μg of protein per sample was electrophoresed in a 4% to 20% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Invitrogen) and electroblotted to polyvinylidene fluoride membrane (Millipore, Billerica, MA). After blocking with 5% skim milk, the membranes were incubated with a rabbit polyclonal antibody against CD14 (1:200, Santa Cruz Biotechnology) or GAPDH antibody (1:1000; Cell Signaling, Danvers, MA) at room temperature for 1 hour. The membranes were washed three times (5 minutes each time) with Tris buffered saline (TBS)/tween and incubated for 30 minutes at room temperature with horseradish peroxidase-labeled anti-rabbit secondary antibody (1:20,000; Jackson ImmunoResearch, West Grove, PA). The membranes were washed three times (5 minutes each time) in TBS/tween, and the proteins were visualized (ECL plus; GE Health Care, Buckinghamshire, UK).
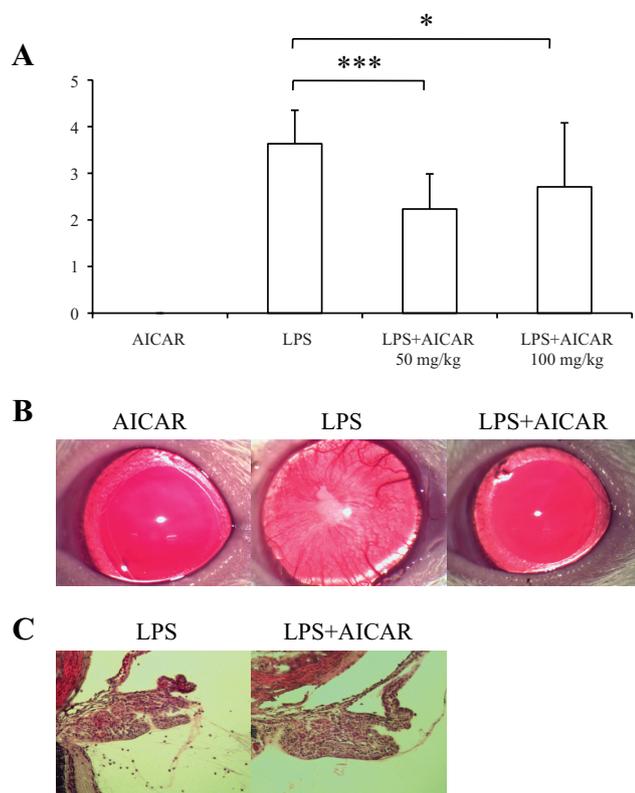
A

B

AICAR   LPS   LPS+AICAR

C

LPS   LPS+AICAR

FIGURE 1. Effect of AICAR on clinical EIU in rats. (**A**) Clinical scores of EIU in Lewis rats in the absence and presence of AICAR was determined at 24 hours after LPS injection. (Normal: $n = 6$; EIU: $n = 15$; LPS with AICAR 50 mg/kg: $n = 15$; LPS with AICAR 100 mg/kg: $n = 12$). Data are shown as mean $\pm$ SD; $*P < 0.05$ and $***P < 0.001$. (**B**) Representative picture. Fibrin formation and posterior synechiae were seen less in AICAR-pretreated EIU rats. (**C**) Histopathologic findings of EIU rat's eyes received LPS with and without AICAR 50 mg/kg pretreatment. The number of leukocytes infiltrated around the iris-ciliary body was suppressed in AICAR pretreated rats. Sections were visualized at magnification $\times 200$.

Measurement of CD14 mRNA Expression by Real Time PCR

Total RNA of PBMC was harvested from cells (RNeasy kit; Qiagen, Valencia, CA), and complementary DNA (cDNA) was generated (OligodT primer and Superscript II; Invitrogen) according to the manufacturer's instructions. Real-time PCR was carried out using the following gene expression assays (TaqMan; Applied Biosystems): CD14 (Rn00572656_g1) and actin (Rn00667869_m1). Quantitative expression data were acquired and analyzed (Step One Plus real-time PCR system; Applied Biosystems, Foster City, CA).

Statistical Analysis

All results were expressed as mean $\pm$ SD. EIU scores were compared by the Mann-Whitney test. Continuous variables from the other experiment were analyzed with the unpaired Student's t-test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Amelioration of EIU Treated with AICAR

Figure 1A shows that clinical inflammation scores were significantly reduced in EIU rats pretreated with AICAR 50 mg/kg (2.2 ± 0.75, $n = 15$) and 100 mg/kg (2.7 ± 1.4, $n = 12$) compared with PBS-treated EIU rats (3.6 ± 0.7, $n = 15$) ($P =$

0.00008 and 0.045, respectively). No inflammation was detected in age-matched normal rats that received AICAR alone ($n = 6$). The findings of fibrin formation and posterior synechiae were rarely seen in AICAR-pretreated EIU animals (Fig. 1B). Histopathological findings also revealed severe leukocyte infiltration in EIU rats compared with rats pretreated with AICAR (Fig. 1C). We also performed these experiments with administration of a single dose of AICAR at two other time points: at LPS injection and 6 hours after LPS injection. With these alternative timing regimens, there was no suppression of inflammation compared with controls. EIU clinical scores were 3.7 ± 0.5 ($n = 6$) and 3.9 ± 0.2 ($n = 6$), respectively. Because the pretreatment with AICAR 100 mg/kg dose was not more effective on reducing inflammation than 50 mg/kg (not statistically different, $P = 0.347$), the remainder of the experiments were conducted with the 50 mg/kg dose.

Effects of AICAR on Infiltrating Cells and Protein Concentration in Aqueous Humor

The anti-inflammatory effect of AICAR on anterior chamber infiltrating cells and protein concentration is shown in Figure 2. LPS injection induced severe cell infiltration into the aqueous humor (22.0×10^5 cells/mL, range 2.5–53.0 cells/mL, $n = 11$), whereas the number of cells was reduced with AICAR pretreatment (1.4×10^5 cells/mL, range 0–4.5 cells/mL, $n = 11$, $P = 0.001$). Increased
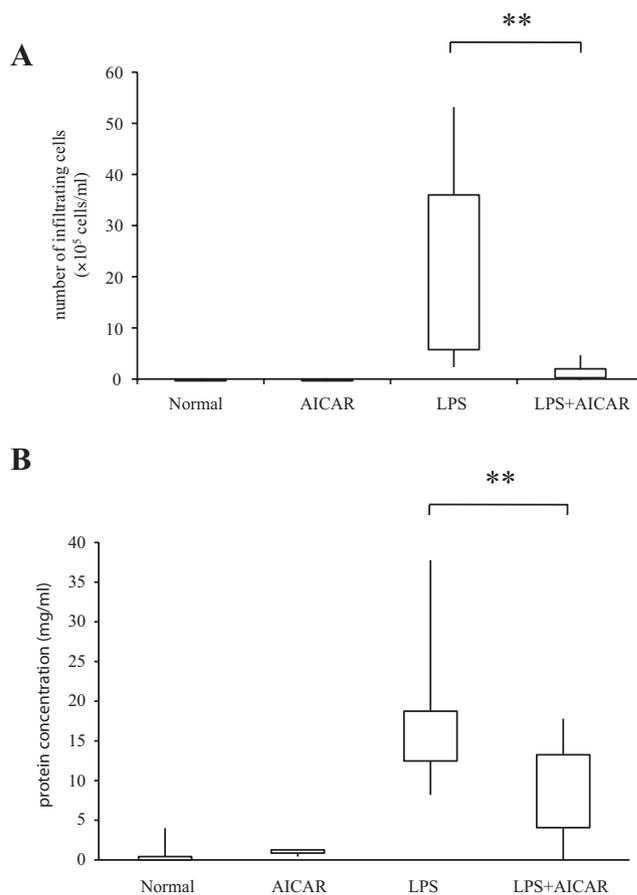
A

B

FIGURE 2. Effect of AICAR on cellular infiltration and protein leakage in the aqueous humor. (**A**) The number of infiltrated cells was determined by cell counting. (**B**) Total protein concentration in the aqueous humor was measured (normal: $n = 7$; AICAR: $n = 6$; LPS: $n = 11$; LPS with AICAR: $n = 11$). LPS-induced cellular infiltration and protein leakage in the aqueous humor which were significantly suppressed by AICAR pretreatment. Data are shown as mean $\pm$ SD; $**P < 0.01$.
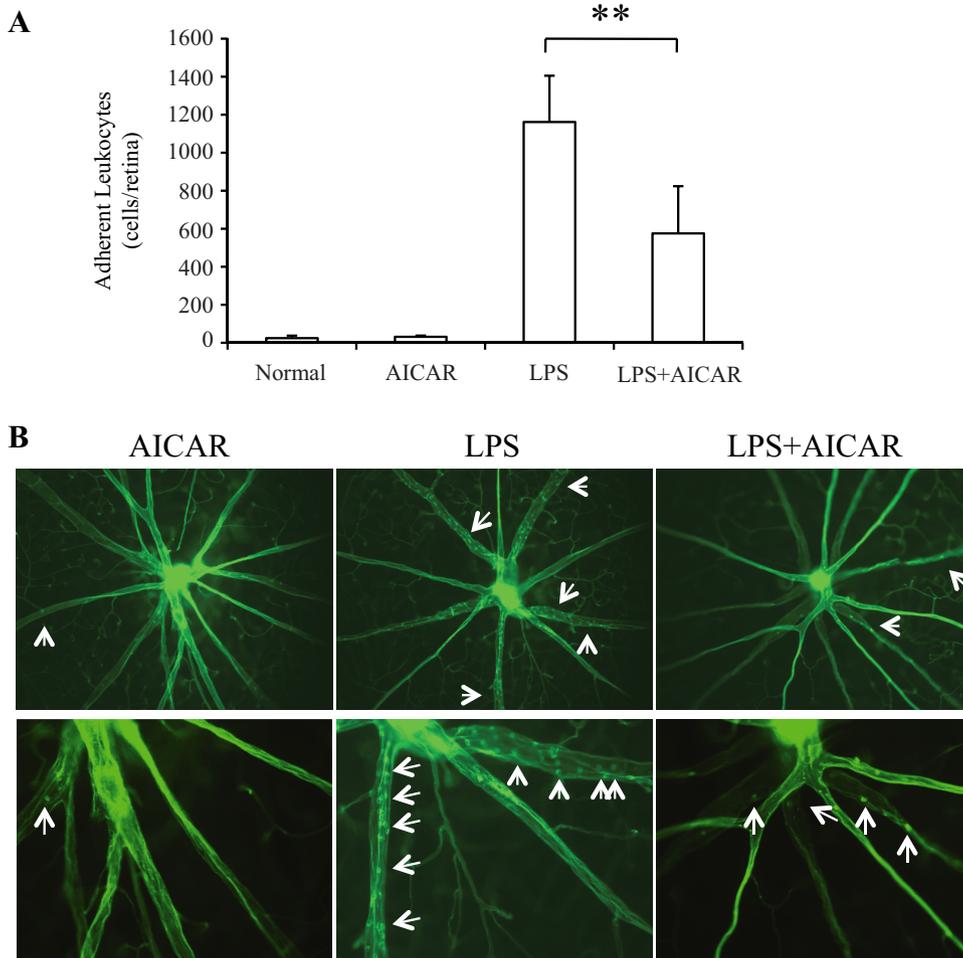
A

B

FIGURE 3. Effect of AICAR on leukocyte adhesion in retinal vessels. (A) The number of retinal adherent leukocytes (normal: $n = 5$; AICAR: $n = 6$; LPS: $n = 8$; LPS with AICAR: $n = 11$). AICAR-pretreated EIU rats showed significantly fewer adherent leukocytes than PBS-treated rats. (B) Flat-mounted retinas from normal controls with AICAR, LPS only, and LPS with AICAR pretreatment. EIU rats revealed significant number of leukocyte adhesion (*arrows*) compared with AICAR pretreated rats. Data are shown as mean ± SD; **$P < 0.01$.

total protein concentration in aqueous humor in EIU rats (18.3 mg/mL, range 8.2–37.8 cells/mL, $n = 11$) was also inhibited in AICAR-pretreated EIU rats (7.9 mg/mL, range 0–17.8 cells/mL, $n = 11$, $P = 0.006$). There were no significant infiltrated cells or protein levels observed in the normal controls ($n = 6$) and AICAR-only treated rats ($n = 6$).

Effects of AICAR on Retinal Leukocyte Adhesion

EIU-associated leukocyte adhesion to retinal vascular endothelium was also reduced with AICAR pretreatment (Fig. 3A). Normal controls ($n = 5$) and AICAR-only treated rats ($n = 6$) showed few adherent leukocytes. In contrast, EIU rats revealed significant numbers of adherent leukocytes at 24 hours after LPS injection (1163 ± 243.6 cells, $n = 8$). AICAR pretreatment of EIU rats resulted in significant suppression of leukocyte adhesion (575.5 ± 248.5 cells, $n = 11$, $P = 0.001$).

Effects of AICAR on Retinal Vascular Leakage

EIU rats had a higher amount of protein leakage from the retinal vessels as measured with bBSA assay (30.5 ± 21.2 ng/mg total retinal protein, $n = 8$) when compared with AICAR-pretreated EIU rats (11.2 ± 7.2 ng/mg total retinal protein, $n = 8$, $P = 0.03$).

Effects of AICAR on NF-κB Activity

To investigate the LPS-induced signal transduction pathway, P65 protein levels translocated into the nuclei of retinal cell extracts were examined 3 hours after LPS injection. As shown in Figure 4, protein levels in the nuclear extracts were signif-

icantly elevated in PBS-treated EIU rats (27.3 ± 11.0 ng/mg total protein, $n = 9$) compared with control rats (8.3 ± 8.6 ng/mg total protein, $n = 6$, $P = 0.004$), whereas AICAR pretreatment significantly suppressed the upregulation (18.2 ± 3.9 ng/mg total protein, $n = 9$, $P = 0.03$).
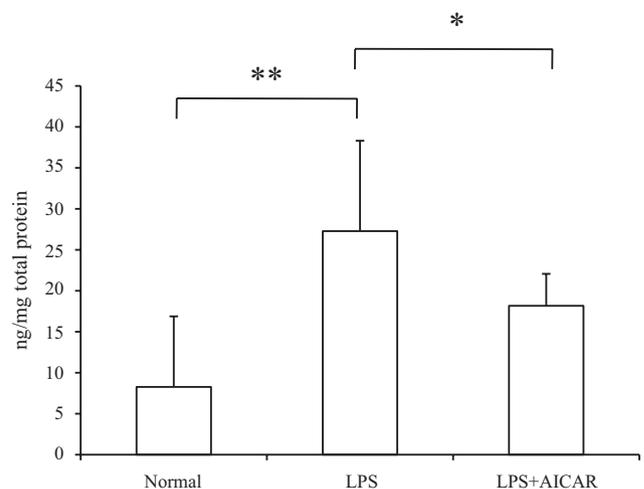

FIGURE 4. Effect of AICAR on NF-κB activity. NF-κB P65 levels in the retinal nuclear extract from normal control ($n = 6$), LPS ($n = 9$), LPS with AICAR ($n = 9$) were determined by ELISA. Data are shown as mean ± SD; *$P < 0.05$, **$P < 0.01$.

TABLE 1. Cytokine and Chemokine Production in Aqueous Humor, Retina, and Serum

	LPS	LPS with AICAR	P
Aqueous Humor (n = 12)			
MCP-1, ng/mL	4.4 ± 3.9	0.8 ± 1.0	0.005
TNF-α, pg/mL	277.8 ± 123.9	92.5 ± 140.5	0.002
ICAM-1, ng/mL	8.7 ± 4.4	4.4 ± 3.3	0.037
Retina (n = 14, pg/total retinal protein)			
MCP-1	18.7 ± 11.3	8.8 ± 2.9	0.004
TNF-α	ND	ND	
ICAM-1	316.4 ± 132.3	202.9 ± 113.4	0.021
Serum (n = 11)			
MCP-1, ng/mL	6.8 ± 0.5	0.4 ± 0.2	0.002
TNF-α, pg/mL	147.2 ± 337.1	57.8 ± 143.1	0.475
ICAM-1, ng/mL	28.7 ± 8.1	21.6 ± 5.0	0.023

ND, not detectable.

Effects of AICAR on Cytokine and Chemokine Production in Aqueous Humor and Retina

The effects of AICAR on intraocular cytokine and chemokine levels were examined by ELISA (Table 1). Protein expression of CCL-2/MCP-1, TNF-α, and ICAM-1 in the aqueous humor (n = 12) and retina (n = 14) were measured. When compared with PBS-treated EIU rats, AICAR-pretreated EIU rats had significantly lower aqueous humor levels of CCL-2/MCP-1 (P = 0.005), TNF-α (P = 0.002), and ICAM-1 (P = 0.04). Similarly, expression of CCL-2/MCP-1 and ICAM-1 in the retina was suppressed in the AICAR-pretreated group as compared with the PBS-treated group (P = 0.004 and P = 0.02, respectively). TNF-α protein was not detected in any retina.

Effects of AICAR on Serum Cytokine and Chemokine and CD14 Expression in PBMCs

Because AICAR was administered systemically, its suppressing effects on the intraocular inflammation could have been mediated locally or systemically. To investigate the systemic effects of AICAR during EIU development, we measured the serum cytokine levels 3 hours after LPS injection (n = 11). Both CCL-2/MCP-1 and ICAM-1 levels were suppressed in AICAR-pretreated EIU rats compared with PBS-treated rats (CCL-2/MCP-1: 0.4 ± 0.2 ng/mL vs. 6.8 ± 0.5 ng/mL, P = 0.002 and ICAM-1: 21.6 ± 5.0 ng/mL vs. 28.7 ± 8.1 ng/mL, P = 0.02).

LPS is thought to mediate its effects through the CD14 receptor on leukocytes. We thus examined the effects of AICAR on the expression of both mRNA and protein expression of CD14 in PBMC by real time PCR and Western blot (Fig. 5). Three hours after LPS injection, CD14 mRNA expression in PBMCs from EIU rats was significantly increased (3.8 ± 0.7, n = 9) compared with normal rats (0.7 ± 0.3, n = 6, P = 0.009). AICAR pretreatment significantly suppressed CD14 mRNA levels (1.7 ± 1.0, n = 9, P = 0.03). Similarly, the protein expression of CD14 was increased in EIU rats and decreased in AICAR-pretreated rats.

DISCUSSION

AICAR administration suppresses ocular inflammation in this EIU-model as measured by slit lamp assessment and histopathology. Anterior chamber cell infiltration, protein concentration, and the expression of proinflammatory cytokines and chemokines (MCP-1, TNF-α, and ICAM-1) were also reduced by AICAR pretreatment. To our best knowledge, this is the first report of AICAR's suppressive effect on ocular inflammation in vivo.

In addition to AICAR's suppression of the acute anterior inflammatory process, we also found AICAR pretreatment re-

duces posterior segment inflammation in this model. Leukocyte adhesion to the retinal vessel is a well documented finding in EIU and expression of adhesion molecules such as ICAM-1 play a pivotal role in the pathogenesis of this finding.[33] It has been previously noted that ICAM-1 is expressed on vascular endothelial cell of the iris and the ciliary body and that an antibody to ICAM-1 reduces ocular inflammation.[9] In this study, the number of adherent leukocytes in retinal vessels of EIU rats was reduced by AICAR pretreatment. This is explained at least in part by the observed reduction in retinal expression of ICAM-1 and MCP-1, which is also involved in leukocyte recruitment.[34] Moreover, retinal vascular impermeability is well maintained in the AICAR-pretreated rats. This can also be at least partially attributed to the reduced expression of inflam-
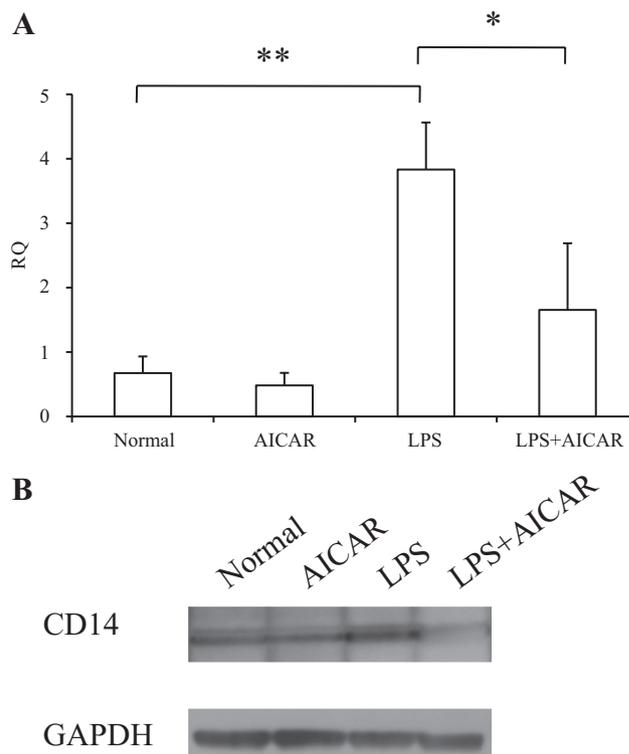

FIGURE 5. Effect of AICAR on CD14 expression in PBMC. (A) mRNA expression (normal: n = 6; AICAR: n = 6; LPS: n = 9; LPS with AICAR: n = 9) was measured by ELISA. Data are shown as mean ± SD; *P < 0.05 and **P < 0.01. (B) Protein levels of CD14 were determined by Western blot analysis. Data are representative of three independent experiments with similar results.

matory mediators observed in our study. These results suggest that AICAR could be effective for the posterior segment manifestations of inflammation, such as cystoid macular edema, which are a significant cause of vision loss in human uveitis.[1,35]

We also investigated AICAR's effect on NF-κB activity in the eye, another potential pathway of its anti-inflammatory effect. Stimulation by various signals, including exposure to LPS, led to NF-κB activation and its movement into the nucleus where it triggers transcription of various proinflammatory genes including MCP-1, TNF-α, and ICAM-1.[36,37] In the retinal samples, LPS-induced NF-κB translocation to nuclei was significantly reduced in AICAR-pretreated rats. Our results suggest that subsequent diminished expression of inflammatory mediators in the eye could be the result of inhibition of NF-κB activity.

Because AICAR was administered systemically, its anti-inflammatory effects on EIU may be mediated partially through systemic changes. Similar to previous reports of its use, AICAR reduced the serum inflammatory cytokine and chemokine production. We next examined CD14 expression in PBMCs. It has been reported that LPS stimulation elevates expression of CD14, which is necessary for LPS recognition.[15,38] In this study, both protein and mRNA expression of CD14 were increased after LPS stimulation and pretreatment with AICAR significantly suppressed these elevations. It has been reported that LPS stimulation decreases AMPK activity in macrophages and increases their production of inflammatory cytokines, while AICAR may upregulate the AMPK activity and suppress cytokine production.[39] One of the treatment effects of AICAR in this study is considered to decrease LPS susceptibility by suppressing CD14 expression.

AICAR inhibits NF-κB signaling[25,26,28] through AMPK activation[40] and we have shown that AICAR affects NF-κB signaling in the eye; this may indicate a direct effect of AICAR on EIU. Some investigators have previously shown that some effects of AICAR are independent of AMPK activation.[30,41-43] In this study, we did not specifically examine AMPK activity in the eye and it is a possibility that part of the treatment effect of AICAR shown in our study is independent of AMPK activity in the eye. Future investigations are needed to further clarify the mechanism of AICAR's effect on ocular inflammation.

These initial results indicate that systemic administration of AICAR reduced the severity of EIU by suppressing the systemic reaction to LPS and local inflammatory cytokine production partially through the NF-κB pathway. In this study, AICAR's effect was confirmed only when it was used as pretreatment, suggesting AICAR might be effective for preventing the relapse of intraocular inflammation. AICAR has proven to be efficacious and well-tolerated in human clinical trials of myocardial infarction,[22] diabetes,[23] and leukemia.[24] If our findings are further validated, AICAR may warrant investigation as a novel therapeutic strategy for human uveitis.

References

1. Durrani OM, Tehrani NN, Marr JE, Moradi P, Stavrou P, Murray PI. Degree, duration, and causes of visual loss in uveitis. *Br J Ophthalmol.* 2004;88:1159–1162.

2. Durrani OM, Meads CA, Murray PI. Uveitis: a potentially blinding disease. *Ophthalmologica.* 2004;218:223–236.

3. Galor A, Jabs DA, Leder HA, et al. Comparison of antimetabolite drugs as corticosteroid-sparing therapy for noninfectious ocular inflammation. *Ophthalmology.* 2008;115:1826–1832.

4. Imrie FR, Dick AD. Biologics in the treatment of uveitis. *Curr Opin Ophthalmol.* 2007;18:481–486.

5. Rosenbaum JT, McDevitt HO, Guss RB, Egbert PR. Endotoxin-induced uveitis in rats as a model for human disease. *Nature.* 1980;286:611–613.

6. Hoekzema R, Murray PI, van Haren MA, Helle M, Kijlstra A. Analysis of interleukin-6 in endotoxin-induced uveitis. *Invest Ophthalmol Vis Sci.* 1991;32:88–95.

7. Koizumi K, Poulaki V, Doehmen S, et al. Contribution of TNF-alpha to leukocyte adhesion, vascular leakage, and apoptotic cell death in endotoxin-induced uveitis in vivo. *Invest Ophthalmol Vis Sci.* 2003;44:2184–2191.

8. Tuaillon N, Shen DF, Berger RB, Lu B, Rollins BJ, Chan CC. MCP-1 expression in endotoxin-induced uveitis. *Invest Ophthalmol Vis Sci.* 2002;43:1493–1498.

9. Becker MD, Garman K, Whitcup SM, Planck SR, Rosenbaum JT. Inhibition of leukocyte sticking and infiltration, but not rolling, by antibodies to ICAM-1 and LFA-1 in murine endotoxin-induced uveitis. *Invest Ophthalmol Vis Sci.* 2001;42:2563–2566.

10. Trichonas G, Manola A, Morizane Y, et al. A novel nonradioactive method to evaluate vascular barrier breakdown and leakage. *Invest Ophthalmol Vis Sci.* 2010;51:1677–1682.

11. Herbort CP, Okumura A, Mochizuki M. Immunopharmacological analysis of endotoxin-induced uveitis in the rat. *Exp Eye Res.* 1989;48:693–705.

12. Avunduk MC, Avunduk AM, Oztekin E, Baltaci AK, Ozyazgan Y, Mogolkoc R. Etanercept treatment in the endotoxin-induced uveitis of rats. *Exp Eye Res.* 2004;79:357–365.

13. Chang JH, McCluskey PJ, Wakefield D. Toll-like receptors in ocular immunity and the immunopathogenesis of inflammatory eye disease. *Br J Ophthalmol.* 2006;90:103–108.

14. Wakefield D, Gray P, Chang J, Di Girolamo N, McCluskey P. The role of PAMPs and DAMPs in the pathogenesis of acute and recurrent anterior uveitis. *Br J Ophthalmol.* 2010;94:271–274.

15. Takeda K, Kaisho T, Akira S. Toll-like receptors. *Annu Rev Immunol.* 2003;21:335–376.

16. Sanchez-Lemus E, Benicky J, Pavel J, et al. Angiotensin II AT1 blockade reduces the lipopolysaccharide-induced innate immune response in rat spleen. *Am J Physiol Regul Integr Comp Physiol.* 2009;296:R1376–R1384.

17. Poulaki V, Iliaki E, Mitsiades N, et al. Inhibition of Hsp90 attenuates inflammation in endotoxin-induced uveitis. *FASEB J.* 2007;21:2113–2123.

18. Hardie DG, Carling D. The AMP-activated protein kinase—fuel gauge of the mammalian cell? *Eur J Biochem.* 1997;246:259–273.

19. Hardie DG, Carling D, Carlson M. The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? *Annu Rev Biochem.* 1998;67:821–855.

20. Winder WW, Hardie DG. AMP-activated protein kinase, a metabolic master switch: possible roles in type 2 diabetes. *Am J Physiol.* 1999;277:E1–E10.

21. Narkar VA, Downes M, Yu RT, et al. AMPK and PPARdelta agonists are exercise mimetics. *Cell.* 2008;134:405–415.

22. Mangano DT, Miao Y, Tudor IC, Dietzel C. Post-reperfusion myocardial infarction: long-term survival improvement using adenosine regulation with acadesine. *J Am Coll Cardiol.* 2006;48:206–214.

23. Cuthbertson DJ, Babraj JA, Mustard KJ, et al. 5-aminoimidazole-4-carboxamide 1-beta-D-ribofuranoside acutely stimulates skeletal muscle 2-deoxyglucose uptake in healthy men. *Diabetes.* 2007;56:2078–2084.

24. Campas C, Lopez JM, Santidrian AF, et al. Acadesine activates AMPK and induces apoptosis in B-cell chronic lymphocytic leukemia cells but not in T lymphocytes. *Blood.* 2003;101:3674–3680.

25. Giri S, Nath N, Smith B, Viollet B, Singh AK, Singh I. 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside inhibits proinflammatory response in glial cells: a possible role of AMP-activated protein kinase. *J Neurosci.* 2004;24:479–487.

26. Zhao X, Zmijewski JW, Lorne E, et al. Activation of AMPK attenuates neutrophil proinflammatory activity and decreases the severity of acute lung injury. *Am J Physiol Lung Cell Mol Physiol.* 2008;295:L497–L504.

27. Nath N, Giri S, Prasad R, Salem ML, Singh AK, Singh I. 5-aminoimidazole-4-carboxamide ribonucleoside: a novel immunomodulator with therapeutic efficacy in experimental autoimmune encephalomyelitis. *J Immunol.* 2005;175:566–574.

28. Prasad R, Giri S, Nath N, Singh I, Singh AK. 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside attenuates experimental au-

toimmune encephalomyelitis via modulation of endothelial-mono-cyte interaction. *J Neurosci Res.* 2006;84:614–625.

29. Theodoropoulou S, Kolovou PE, Morizane Y, et al. Retinoblastoma cells are inhibited by aminoimidazole carboxamide ribonucleotide (AICAR) partially through activation of AMP-dependent kinase. *FASEB J.* 2010;24:2620–2630.

30. Qin S, Ni M, De Vries GW. Implication of S-adenosylhomocysteine hydrolase in inhibition of TNF-alpha- and IL-1beta-induced expression of inflammatory mediators by AICAR in RPE cells. *Invest Ophthalmol Vis Sci.* 2008;49:1274–1281.

31. Pouvreau I, Zech JC, Thillaye-Goldenberg B, Naud MC, Van Rooijen N, de Kozak Y. Effect of macrophage depletion by liposomes containing dichloromethylene-diphosphonate on endotoxin-induced uveitis. *J Neuroimmunol.* 1998;86:171–181.

32. Smith LE, Wesolowski E, McLellan A, et al. Oxygen-induced retinopathy in the mouse. *Invest Ophthalmol Vis Sci.* 1994;35:101–111.

33. Yang L, Froio RM, Sciuto TE, Dvorak AM, Alon R, Luscinskas FW. ICAM-1 regulates neutrophil adhesion and transcellular migration of TNF-alpha-activated vascular endothelium under flow. *Blood.* 2005;106:584–592.

34. Fuentes ME, Durham SK, Swerdel MR, et al. Controlled recruitment of monocytes and macrophages to specific organs through transgenic expression of monocyte chemoattractant protein-1. *J Immunol.* 1995;155:5769–5776.

35. Lardenoye CW, van Kooij B, Rothova A. Impact of macular edema on visual acuity in uveitis. *Ophthalmology.* 2006;113:1446–1449.

36. Baeuerle PA, Henkel T. Function and activation of NF-kappa B in the immune system. *Annu Rev Immunol.* 1994;12:141–179.

37. Baldwin AS Jr. The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu Rev Immunol.* 1996;14:649–683.

38. Landmann R, Knopf HP, Link S, Sansano S, Schumann R, Zimmerli W. Human monocyte CD14 is upregulated by lipopolysaccharide. *Infect Immun.* 1996;64:1762–1769.

39. Sag D, Carling D, Stout RD, Suttles J. Adenosine 5′-monophosphate-activated protein kinase promotes macrophage polarization to an anti-inflammatory functional phenotype. *J Immunol.* 2008; 181:8633–8641.

40. Katerelos M, Mudge SJ, Stapleton D, et al. 5-aminoimidazole-4-carboxamide ribonucleoside and AMP-activated protein kinase inhibit signalling through NF-kappaB. *Immunol Cell Biol.* 2010;88: 754–760.

41. Jhun BS, Jin Q, Oh YT, et al. 5-Aminoimidazole-4-carboxamide riboside suppresses lipopolysaccharide-induced TNF-alpha production through inhibition of phosphatidylinositol 3-kinase/Akt activation in RAW 264.7 murine macrophages. *Biochem Biophys Res Commun.* 2004;318:372–380.

42. Kuo CL, Ho FM, Chang MY, Prakash E, Lin WW. Inhibition of lipopolysaccharide-induced inducible nitric oxide synthase and cyclooxygenase-2 gene expression by 5-aminoimidazole-4-carboxamide riboside is independent of AMP-activated protein kinase. *J Cell Biochem.* 2008;103:931–940.

43. Labuzek K, Liber S, Gabryel B, Okopien B. AICAR (5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside) increases the production of toxic molecules and affects the profile of cytokines release in LPS-stimulated rat primary microglial cultures. *Neurotoxicology.* 2010;31:134–146.