Topical administration of diminazene aceturate decreases inflammation in endotoxin-induced uveitis

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Purpose: Our previous study demonstrated that an intraperitoneal injection of Diminazene Aceturate (DIZE) attenuated uveitis by activating ocular angiotensin-converting enzyme 2 (ACE2). Here, we investigated the anti-inflammatory effects on the ocular anterior segment of a topical administration of a DIZE solution and explored the downstream target molecules involved in the anti-inflammatory mechanism after ACE2 activation.

Methods: Endotoxin-induced uveitis (EIU) in rats was induced by a subcutaneous injection of lipopolysaccharides (LPS, 200 μg) in 0.1 ml of sterile saline. DIZE (0.025, 0.05, or 0.1%) and dexamethasone (0.1%) solutions were applied topically (10 μl eyedrops) to both eyes 6X every two hours before and after LPS injection. The inflammation of the ocular anterior segment was observed and the clinical scores were evaluated 24 h after LPS injection. The total protein concentration and levels of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in the aqueous humor were determined. CD11b-positive cells adjacent to the iris ciliary body (ICB) were stained by immunohistochemistry. The mRNA levels of inflammatory cytokines and mediators, including IL-1β, TNF-α, COX-2, and iNOS or NF-κB subunit p65 in the ICB, were analyzed by real time RT–PCR. The protein expression of NF-κB p65 and the phosphorylated protein of p38 MAPK were detected by western blotting.

Results: A topical administration of DIZE decreased clinical scores and the total protein concentration, as well as TNF-α and IL-6 levels in the aqueous humor. Meanwhile, the mRNA levels of inflammatory cytokines and mediators, including IL-1β, TNF-α, COX-2, and iNOS in the ICB, were downregulated. DIZE reduced the recruitment of CD11b-positive cells adjacent to the ICB. Furthermore, DIZE downregulated the expressions of NF-κB subunit p65 at protein and mRNA levels and inhibited the phosphorylation of p38 MAPK protein in the ICB.

Conclusions: A topical administration of DIZE suppressed ocular inflammation in EIU and decreased the levels of inflammatory cytokines. DIZE attenuated the activation of NF-κB and p38 MAPK in EIU, which may be associated with ACE2-mediated anti-inflammatory effects. Our data provided further evidence that DIZE may represent a novel class of drug for the management of ocular inflammation.

Uveitis, a common inflammatory ocular condition, leads damage of the uveal tract and causes blindness [1]. In clinical service, the most commonly used drugs for treating uveitis are corticosteroids and immunosuppressors. However, these drugs cause many systemic and local side effects. Therefore, it is desirable to search for novel remedies.

Diminazene aceturate (DIZE), an aromatic diamidine, has been used for treating trypanosomiasis, babesiosis, and other protozoal diseases in livestock since 1955. Recently, researchers found DIZE presented anti-inflammatory properties besides its anti-protozoal effect. DIZE decreased the levels of pro-inflammatory cytokines produced by splenetic and hepatic macrophages in vitro [2]. It was suggested that DIZE treatment also inhibited IL-1β and TNF-α expressions in myocardial infarction [3]. In addition, the anti-inflammatory properties may be associated with the inhibition of the phosphorylation of mitogen-activated protein kinases (MAPKs) and signal transducers and activators of transcription (STAT) proteins [4]. We found DIZE increased endogenous angiotensin-converting enzyme 2 (ACE2) activity and exerted a protective role in endotoxin-induced uveitis (EIU) [5]. These results suggested DIZE was promising to become a novel drug for treating ocular inflammatory diseases. However, the downstream target molecules involved in the anti-inflammatory mechanism after ACE2 activation remain unclear.

Nuclear factor (NF)-κB and MAPK family signal pathways cooperatively upregulate the pro-inflammatory cytokine gene expression [6]. The activation of NF-κB plays a pivotal role in the pathogenesis of EIU and the NF-κB inhibitor decreased inflammation in uveitis [7,8]. The MAPK family, including extracellular signal-regulated kinases (ERKs), c-jun N-terminal kinases (JNKs), and p38 MAPK, controls a vast array of physiologic processes. Although these enzymes are regulated by phosphorylation and are activated as a cascade, the three major sub-groups have different
properties: ERKs control cell division, JNKs regulate transcription, and p38 MAPK affects inflammatory responses [9].

EIU is an animal model of acute ocular anterior segment inflammation [10]. Among the prominent characteristics of EIU are infiltration of inflammatory cells (neutrophils, monocytes, and dendritic cells) into the eye anterior segment and leakage of protein into the aqueous humor [11,12]. The exact mechanism of EIU remains not well understood. However, evidence indicates the elevated expressions of cytokines, including pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β), cyclooxygenase-2 (COX-2), and inducible nitric-oxide synthase (iNOS), play an important role in the development of EIU [12-14]. In addition, previous studies showed that infiltrating inflammatory cells in iris ciliary body (ICB) is partially responsible for the production of these cytokines [15,16]. In this study, we investigated the anti-inflammatory effects of the topical administration of DIZE on the ocular anterior segment in EIU rats and explored the underlying mechanism.

METHODS

Animals: Female Sprague-Dawley rats weighing 180–220 g were purchased from the Laboratory Animal Center of Chongqing Medical University (Chongqing, China). All rats were kept in a standard animal room and exposed to a 12 h:12 h light-dark cycle. The study protocol was approved by the Animal Ethics Committee of the First Affiliated Hospital of Chongqing Medical University. Every effort was made to minimize animal discomfort and stress. All experiments were conducted in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Reagents: Lipopolysaccharides (LPS, L2880) from Escherichia coli 055B5 were obtained from Sigma-Aldrich Chemical Co. (St Louis, MO). DIZE was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Dexamethasone (Dexa) was obtained from Shanghai Yuanye Bio-Technology Co. (Shanghai, China).

Induction of EIU and DIZE eye drops application: EIU was induced by subcutaneous injection of LPS (200 μg) dissolved in 0.1 ml of sterile saline in the thigh [17]. Control animals received the same volume of pyrogen-free saline. DIZE (0.025, 0.05, and 0.1%) or 0.1% Dexa were applied topically to both eyes of rats 6X every two hours before and after LPS injection, respectively. Each eye received 10 μl of eyedrops each time. The control animals received vehicles following the same procedure.

Clinical score and protein concentration in the aqueous humor: Animals were anesthetized 24 h after LPS injection, and the clinical score of intraocular inflammation was examined by slit lamp biomicroscopy (n = 8 per group). The clinical severity of ocular inflammation was graded by two independent observers according to a scoring system (Table 1) [5,18].

Aqueous humor samples were collected from both eyes with a 30-gauge needle under a microscope and were stored in ice water until testing. The total protein concentration in the aqueous humor was determined by a bicinchoninic acid (BCA) protein assay kit (Beyotime Institute of Biotechnology, Jiangsu, China) on the same day of sample collection (n = 6 per group).

TNF-α and IL-6 proteins in the aqueous humor: The protein levels of TNF-α and IL-6 in the aqueous humor were assessed using an enzyme-linked immunosorbent assay kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s instruction. The aqueous humor samples from both eyes of one animal were pooled and diluted with assay buffer. Equal amounts of the aqueous humors were used for the measurement of TNF-α and IL-6 (n = 4 per group).

Real-time quantitative PCR analysis: At 24 h after LPS injection, rats were killed with an overdose of anesthesia, and the eyes were immediately enucleated. The ICB complex was carefully isolated under a surgical microscope. Total ICB RNA was extracted from tissues using TRIzol (Invitrogen, Paisley, UK) following the manufacturer’s instructions. RNA concentrations were tested using a Nano instrument.
(NanoDrop Technologies, Wilmington, DE). In addition, cDNA was generated using the PrimeScript® RT reagent Kit (Takara Biotechnology, Dalian, China) according to the manufacturer’s instructions [19]. Real-time quantitative PCR was performed on Applied Biosystems Prism 7500 (Life Technologies Co., CA). Samples underwent 40 cycles of amplification in a volume of 20 µl using the all-in-one qPCR Mix (GeneCopoeia Inc., Rockville, MD). The conditions were 95 °C for 10 min, followed by 40 cycles of 10 s at 95 °C, 20 s at 60 °C and 15 s at 72 °C. Primers used to analyze TNF-α, IL-1β, iNOS, COX-2, NF-κB p65, and GAPDH expressions are summarized in Table 2. The relative amount of target mRNA was calculated from the obtained ΔCt values for the target and endogenous reference gene GAPDH using the 2^ΔΔCt cycle threshold method. All samples were analyzed in triplicate (n = 4 per group).

**Immunohistochemistry:** Rats were sacrificed 24 h after LPS administration. Eyes were enucleated and fixed in 4% paraformaldehyde for 12 h and embedded in paraffin. Serial sagittal sections (4 µm) were cut through the cornea-optic nerve head. Paraffin-embedded serial sections were deparaffinized in xylene, rehydrated through a graded ethanol series, and washed with PBS. Antigen retrieval was performed by heating sections in a microwave oven in citrate buffer (0.01 mol/l, pH 6.0) for 15 min. Endogenous peroxidase was eliminated by incubation in 3% hydrogen peroxide in PBS for 30 min, and the sections were washed in PBS for 5 min 3X and incubated in 10% goat serum for 30 min. The sections were incubated with antibodies (dilution 1:200, ABCAM, Cambridge, UK) against CD11b overnight at 4 °C and stained with peroxidase. The numbers of CD11b-positive cells adjacent to the ICB were counted and averaged from six slides (n = 6 per group).

**Western blotting analysis:** The ICB complex was homogenized and solubilized in a cell lysis solution containing 1% protease inhibitors [19]. The total protein concentration was determined by the BCA protein assay kit. In addition, 60 µg of protein lysate was resolved in 10% SDS–PAGE and then transferred onto nitrocellulose membranes (Millipore, Billerica, MA). The blots of non-phosphorylation and phosphorylation were blocked with 5% non-fat dry milk or 5% BSA, respectively, and probed overnight at 4 °C with primary antibodies specific to NF-κB p65 (1:1200; ABCAM, Cambridge, UK), phosphorylated p38 MAPK(1:1000; ABCAM, Cambridge, UK), and p38 MAPK(1:1000; ABCAM, Cambridge, UK). The blots were washed with Tris-buffered saline with Tween-20 and incubated with an HRP-conjugated secondary antibody for 1 h at 37 °C. Blots were developed using enhanced chemiluminescence (Amersham, Piscataway, NJ). Bands were analyzed using Image J software (Version1.43, Broken Symmetry Software, Bethesda, MD). An NF-κB p65 analysis was normalized against a housekeeping protein β-actin and a phosphorylated p38 MAPK analysis was normalized against p38 MAPK. The measurements were repeated 3X in each experiment (n = 4 per group).

**Statistical analysis:** Data were presented as mean ± SD. A statistical analysis was performed with GraphPad Prism (GraphPad Software, Inc., San Diego, CA). A one-way analysis of variance (ANOVA) followed by a Bonferroni correction were applied for multiple comparisons. A Mann–Whitney U test was used to compare the EAU score. A p value less than 0.05 was considered statistically significant.

**RESULTS**

**DIZE decreased clinical score and protein concentration in aqueous humor:** We evaluated the effect of DIZE eyedrops on ocular anterior segment inflammation and compared its effect with Dexa solution. At 24 h after LPS injection, fibrinous pupillary membrane and severe iris hyperemia were observed (Figure 1B). The clinical scores in the LPS groups were 3.12±0.83. Compared to the LPS groups, the clinical scores were reduced in both the 0.05% DIZE and the 0.1% DIZE groups (2.12±0.64, p<0.01; 2.12±0.35, p<0.01, respectively), especially in the 0.1% DIZE group (Figure 1C), which showed clear pupils and mild hyperemia. Rats treated with 0.1% Dexa displayed the minimum clinical scores (1.75±0.46, p<0.001).

The total protein concentration in the aqueous humor in the control group was 2.7±0.5 mg/ml. In addition, 0.05%
and 0.1% DIZE decreased the protein level in the aqueous humor (34.9±3.2 mg/ml, p<0.01; 29.1±3.2 mg/ml, p<0.001) in contrast with the LPS group (40.2±2.3 mg/ml; Figure 1F). Furthermore, 0.1% Dexa strongly reduced the protein concentration (14.4±2.4 mg/ml, p<0.001) in EIU rats.

**DIZE decreased TNF-α and IL-6 protein levels in the aqueous humor:** In the LPS group, the protein concentrations of TNF-α and IL-6 in the aqueous humor were 165.4±59.6 pg/ml (Figure 2A) and 155.2±12.8 pg/ml (Figure 2B), respectively. As well, 0.1% DIZE eyedrops significantly decreased TNF-α and IL-6 levels (75.4±19.9 pg/ml; p<0.05, 108.3±31.2 pg/ml; p<0.05, respectively).

**DIZE decreased the mRNA expressions of pro-inflammatory cytokines:** We detected the mRNA expressions of IL-1β, TNF-α, COX-2, iNOS, and NF-κB p65. As shown in Figure 3A,B, the mRNA expressions of IL-1β and TNF-α increased significantly in the LPS group when compared to the control group (p<0.001). In the DIZE treated rats, the mRNA expressions of IL-1β (p<0.001) and TNF-α (p<0.01) in ICB were significantly decreased. COX-2, and iNOS, two critical enzymes catalyzing the formation of prostaglandin E₂ (PGE₂) and nitric oxide (NO), are involved in pro-inflammatory mediator production [14,20]. DIZE treatment also inhibited the mRNA elevations of COX-2 (Figure 3C, p<0.05) and iNOS (Figure 3D, p<0.001). NF-κB transcribes genes responsible for various inflammatory markers. LPS increased the expression of NF-κB p65 mRNA in ICB (p<0.05) at 24 h, but DIZE significantly inhibited the NF-κB p65 mRNA expression (Figure 3E, p<0.001).

Figure 1. DIZE alleviated inflammation in the ocular anterior segment in EIU rats. The ocular inflammation was assessed by slit lamp microscopy. Clinical scores were evaluated and the protein level in the aqueous humor was measured at 24 h after LPS injection. **A:** Image of the anterior segment of a control rat. **B:** Image of the anterior segment of an EIU rat showing fibrinous pupillary membrane and severe iris hyperemia (arrows). **C:** Image of the anterior segment of an EIU rat treated with 0.1% DIZE showed clear pupil and mild hyperemia. **D:** Image of the anterior segment of an EIU rat treated with 0.1% Dexa. **E:** The clinical scores were evaluated 24 h after LPS injection according a criteria of EIU (Table 1, n= 8). **F:** The protein levels in the aqueous humor samples from all groups measured 24 h after LPS injection (n = 6). Values represented mean±SD **p<0.01, ***p<0.001.

Figure 2. DIZE decreased protein concentrations of TNF-α and IL-6 in the aqueous humors of EIU rats. Topical instillation of 0.1% DIZE eyedrops decreased the concentrations of TNF-α (A) and IL-6 (B) in the aqueous humors of EIU rats at 24 h after LPS injection. Values represented mean±SD (n = 4). *p<0.05, **p<0.01, ***p<0.001.
DIZE inhibited recruitment of CD11b positive cells: Immunohistochemistry of EIU in rats was evaluated and the number of CD11b-positive cells was counted 24 h after LPS injection. In the control group, there were barely any CD11b-positive cells (Figure 4A). However, a large number of CD11b-positive cells adjacent to the ICB (66±16 cells/section; Figure 4B) was seen in the LPS-injected rats. Compared to the LPS group, the number of CD11b-positive cells adjacent to the ICB decreased in the 0.1% DIZE group and the 0.1% Dexe group (31±15 cells/section; Figure 4C, 11±8 cells/section; Figure 4D, respectively).

DIZE down-regulated the activity of NF-κB p65 and p38 MAPK: We detected NF-κB p65 protein by western blotting. Figure 5A shows that when NF-κB was activated by LPS, the total p65 protein expression in the ICB was elevated in EIU rats, but the elevation was inhibited by DIZE treatment (Figure 5A, p<0.05) at 24 h. MAPK kinases catalyzed the phosphorylation of MAPKs and turned the MAPKs to an active state. The phosphorylation of the p38 MAPK protein was detected and it was highly expressed in EIU but down-regulated by DIZE treatment at 24 h (Figure 5B, p<0.05) in the ICB of EIU rats.

DISCUSSION

In the present study, we found a topical administration of DIZE in EIU rats ameliorated clinical scores and decreased the levels of pro-inflammatory cytokines and protein in the aqueous humor. The release of cytokines by activated inflammatory cells has been implicated in the pathogenesis of EIU. Among these cytokines, TNF-α, IL-1β, and IL-6 play an important role in the process of EIU [12,13,16]. Here, we found DIZE decreased not only the protein levels of TNF-α

![Figure 3. DIZE downregulated mRNA expressions of inflammatory cytokines and NF-κB p65 in EIU rats. The iris ciliary body complexes were used to detect the mRNA of inflammatory cytokines, including IL-1β, TNF-α, COX-2, iNOS, and NF-κB p65. 0.1% DIZE eyedrops inhibited the mRNA expressions of IL-1β (A), TNF-α (B), COX-2 (C), iNOS (D), and NF-κB p65 (E) in EIU rats 24 h after LPS injection. Values represented mean±SD (n=4). *p<0.05, **p<0.01, ***p<0.001.](image-url)
and IL-6 in the aqueous humor, but also the mRNA expressions of TNF-α and IL-1β in the ICB, suggesting DIZE intervened in the inflammatory response and ameliorated EIU manifestation. PGE₂ and NO production mediated by COX-2 and iNOS, respectively, also had a profound effect on inflammation. Increased COX-2- and iNOS-positive cells in the ICB have been reported 24 h after LPS injection [17]. In agreement with the findings, the mRNA expressions of COX-2 and iNOS in the ICB were elevated in the present study. After the administration of DIZE eyedrops, the mRNA expressions of COX-2 and iNOS were inhibited. Thus, the topical application of DIZE suppressed the intraocular inflammation responses in EIU by blocking the expression of inflammatory mediators. The main infiltrating cell types are the neutrophils and the monocytes in EIU [11]. IL-6, TNF-α, and IL-1β may be produced by a wide variety of inflammatory cells, including neutrophils, lymphocytes, and macrophages in EIU [21,22]. The antigen CD11b (OX42) is expressed on the surface of many leukocytes, such as monocytes, neutrophils, and macrophages [23]. Barely any CD11b-positive cells were found in the control group, suggesting CD11b-positive cells were not dominant in the rats under normal conditions. However, in the EIU group, there were markedly infiltrating inflammatory cells and most were CD11b-positive neutrophils [11]. After DIZE treatment, both the number of neutrophils and the levels of the inflammatory cytokines’ mRNA decreased, suggesting DIZE inhibited the recruitment of neutrophils.

Figure 4. DIZE reduced CD11b-positive cell recruitment adjacent to the iris ciliary body in EIU rats. Immunohistochemical staining of the ICB in EIU rats was performed 24 h after LPS injection. A: Image of the ICB of a control rat. B: Image of the ICB of an LPS-injected rat showing a high number of CD11b-positive cells (arrow). C: Image of the ICB of an EIU rat treated with 0.1% DIZE. D: Image of the ICB of an EIU rat treated with 0.1% Dexa. E: Image of the ICB of a negative control rat. Bars: 50 μm. N.D.: not detected. Values represented mean±SD (n = 6). ***p<0.001.
and neutrophils might be at least partially responsible for releasing cytokines in EIU.

It is well known that in several cell types, LPS is the major inducer of redox-sensitive transcription factor NF-κB, which plays a pivotal role in triggering an array of pro-inflammatory genes, such as TNF-α, IL-1β, IL-6, and COX-2 in inflammatory diseases, including endotoxin and autoimmune-induced uveitis [7,24]. Under normal conditions, the p65 subunit of NF-κB binds with its inhibitor IκB to form the IκB-NF-κBp50/p65 trimetric complex [7]. Once the inhibitory protein IκB is degenerated by phosphorylation, the activated NF-κB p65 is freed and can be recognized by the antibody. Exposure of human monocytic THP-1 cells to LPS for up to 24 h resulted in increasing the levels of p65 proteins in the cytosol and nucleus [25]. Moreover, several studies showed the number of NF-κB p65-positive cells gradually increased from 3 h to 24 h in ICB [17,26,27]. Comparable with these findings, we found the NF-κB p65 protein in the LPS group was more highly expressed than in the control group at 24 h in the ICB. DIZE treatment decreased the NF-κB p65 expression and alleviated inflammation in EIU. On the other hand, the inhibition of the phosphorylation of MAPKs also exhibited anti-inflammatory effects [28,29] and DIZE downregulated the phosphorylation of ERKs and p38 MAPK to modulate cytokine production by macrophages [4]. Similarly, we found DIZE inhibited the phosphorylation of p38 MAPK in the ICB in EIU in vivo. In addition, we found DIZE inhibited the expression of NF-κB p65 and phosphorylation of the p38 MAPK protein in the retinal pigment epithelium cells (data not shown) in vitro. Our data indicated DIZE inhibited inflammation by downregulating the activity of NF-κB p65 and p38 MAPK and decreasing cytokines in the ICB.

DIZE was identified as an activator of ACE2 by cleavage of angiotensin II [30]. Recent studies showed that ACE2/Ang-(1–7)/Mas axis countered angiotensin II-mediated inflammation [31,32]. Meanwhile, the ACE2/Ang-(1–7)/Mas axis exerted its beneficial effect by suppressing MAPK- and NF-κB-dependent pathways [32,33]. Furthermore, the infusion of Ang-(1–7) in rats with permanent cerebral ischemia could significantly decrease the protein expression of not only total NF-κB p65 but also TNF-α, IL-1β, and COX-2, and the anti-inflammatory effects were abrogated by a Mas receptor antagonist A-779 [34]. In our previous study, DIZE increased endogenous ACE2 activity in ocular tissue [5]. We found DIZE increased the expression of ACE2 at mRNA and protein levels in the retinal pigment epithelium cells (data not shown) in vitro. In addition, the mRNA expression of the Mas receptor was also detected in the ciliary body [35]. Therefore, the topical administration of DIZE might also decrease the protein expression of NF-κB p65 and inhibit the phosphorylation of p38 MAPK pathways by activating the ACE2/Ang-(1–7)/Mas axis in the ICB.

In this study, the instillation of different concentrations of DIZE as eyedrops showed dose-dependent anti-inflammatory effects. Compared to Dexe eyedrops, hydrophilic DIZE eyedrops showed a modest effect on EIU in this study. As lipophilic drugs have a higher corneal epithelial permeability than hydrophilic drugs [36], it is possible lipophilic DIZE eyedrops might present stronger anti-inflammatory effects. DIZE had some side effects through systemic injections at high doses in livestock [37]. To avoid potential off-target side effects on other organs, a topical administration of DIZE at a lower dose may offer an ideal approach for a therapeutic intervention for uveitis patients than systemic drug administration.
Nevertheless, due to its highly polar molecular characteristics [38], the application of DIZE as eyedrops may be limited. Therefore, studies aimed to improve its permeability and to understand its detailed pharmacokinetics in the eyes are desirable.

In summary, the topical administration of DIZE effectively decreased the inflammation of EIU by inhibiting the NF-κB and p38 MAPK pathways. DIZE may represent a novel class of drug for the management of uveitis.

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