Suppression of Ocular Inflammation in Endotoxin-Induced Uveitis by Inhibiting Nonproteolytic Activation of Prorenin

Shingo Satofuka,1,2 Atsubiro Ichibara,3 Noribiro Nagai,1,2,4 Kenji Yamashiro,5 Takashi Koto,1,2 Hajime Shinoda,1,2 Kousuke Noda,2 Yoko Ozawa,1,2 Makoto Inoue,2 Kazuo Tsubota,2 Fumiaki Suzuki,5 Yuichi Oike,1,4 and Susumu Ishida1,2

PURPOSE. A recent study revealed that angiotensin receptor signaling mediates ocular inflammation and neovascularization. It was also found that prorenin undergoes nonproteolytic activation leading to upregulation of the renin-angiotensin system (RAS) when prorenin receptor interacts specifically with the handle region of prorenin. The purpose of the present study was to elucidate the role of the receptor-dependent nonproteolytic activation of prorenin in ocular inflammation in endotoxin-induced uveitis (EIU).

METHODS. EIU was induced in Long-Evans rats by a single intraperitoneal injection of 100 μg lipopolysaccharide (LPS). Tissue localization of total prorenin, prorenin receptor, and activated prorenin in the EIU retina was examined by immunochemistry. To inhibit the prorenin receptor-mediated upregulation of the RAS, a decoy handle-region peptide (HRP) was intraperitoneally administered 24 hours before and immediately after the injection of LPS. Twenty-four hours after LPS injection, leukocyte adhesion to the retinal vasculature was evaluated with a concanavalin A lectin perfusion-labeling technique. In addition, leukocyte infiltration into the vitreous cavity and protein concentration in the anterior chamber were also measured. Retinal mRNA and protein levels of intercellular adhesion molecule (ICAM)-1, interleukin (IL)-6, and C-C chemokine ligand (CCL) 2/monocyte chemotactic protein (MCP)-1 were examined by RT-PCR and ELISA.

RESULTS. Retinal vessels in rats with EIU were strongly positive for total prorenin, prorenin receptor, and activated prorenin. Systemic treatment with HRP resulted in dose- and time-dependent inhibition of the leukocyte adhesion and infiltration and the protein leakage, all of which were increased by the induction of EIU. Retinal mRNA expression and protein levels of ICAM-1, CCL2/MCP-1 and IL-6, induced in rats with EIU, were also significantly suppressed with application of HRP.

CONCLUSIONS. These findings demonstrate for the first time that nonproteolytically activated prorenin plays a significant role in the development of ocular inflammation in the EIU model. The present study suggests the potential use of HRP, a decoy peptide binding to the prorenin receptor, as a therapeutic agent to reduce ocular inflammation. (Invest Ophthalmol Vis Sci. 2006;47:2686–2692) DOI:10.1167/iovs.05-1458

Endotoxin-induced uveitis (EIU) is an animal model of acute ocular inflammation induced by the administration of lipopolysaccharide (LPS), a component of Gram-negative bacterial outer membranes.1,2 Because uveitis frequently leads to severe vision loss and blindness with retinal vasculitis, retinal detachment, and glaucoma, it is important to elucidate more detailed mechanisms in the development of ocular inflammation. LPS enhances the expression of various inflammatory mediators, such as interleukin (IL)-6,3,4 tumor necrosis factor (TNF)-α,5 and C-C chemokine ligand (CCL) 2/monocyte chemotactic protein (MCP)-1,6 which contribute to the development of EIU, resulting in the breakdown of the blood-ocular barrier and in the infiltration of leukocytes. For the first phase of leukocyte infiltration, cell adhesion to vascular endothelium, in which adhesion molecules play major roles, is essential.7 Among various adhesion molecules, intercellular adhesion molecule (ICAM)-1 and its receptor, lymphocyte function–associated antigen (LFA)-1, are necessary for the development of EIU.7–9 Although EIU was originally used as a model mimicking anterior uveitis, increasing evidence shows EIU as involving inflammation in the posterior segment of the eye with recruitment of leukocytes adhering to the retinal vasculature and infiltrating the vitreous cavity.10,11

Recently, we demonstrated that angiotensin II type 1 receptor (AT1-R) blockers, widely and safely used for anti-hypertensive therapy, have an inhibitory effect on ocular inflammation in the EIU model, which is complicated by upregulated expression of AT1-R on retinal vessels.1,2 This finding is supported by several recent reports showing that the renin-angiotensin system (RAS), originally regarded as an important controller of systemic blood pressure, plays crucial roles in pathologic vascular conditions including inflammation and neovascularization via interaction of angiotensin II with AT1-R.13–16 The initial step for upregulation of the RAS is classically known as proteolytic activation, whereby prorenin is converted to the active (mature) form of renin by the processing enzymes to remove the prorenin prosegment, which folds into an active site cleft of mature renin. Renin is well known to be a rate-limiting enzyme in the RAS for the cleavage of angiotensinogen to angiotensin I, which angiotensin-converting enzyme processes to angiotensin II, a final effector molecule that interacts with its cognate receptors, AT1-R and AT2-R.

In addition to the proteolytic activation of prorenin, non-proteolytic activation of prorenin, which was recently demonstrated in vitro,17 has attracted growing attention as a local upregulator of the RAS, causing organ damage. In the mechanism of nonproteolytic activation, when the prorenin binding proteins interact selectively with the handle region of the prorenin prosegment, prorenin undergoes conformational change with exposure of the active center and obtains enzy-
Nonproteolytic Activation of Prorenin in Ocular Inflammation

**Experimental phenomena** have never been found in vivo. Acidic pH or low-temperature, which are called acid-acti-
tive renin receptor with the handle region of the prorenin prosegment. (B) Preparation of the decoy peptide corresponding to the HRP. Amino acid sequences of the rat prorenin prosegment and HRP.

**METHODS**

**Animals and Induction of EIU**

Long-Evans rats (5–7 weeks old; SLC, Shizuoka, Japan) were used. All animal experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The ethics committee of our institution approved all surgical interventions and animal care procedures, which were in accordance with the Guidelines and Policies for Animal Surgery provided by the Animal Study Committees of the Central Institute for Experimental Animals of Keio University. Animals received a single intraperitoneal injection of 100 µg LPS from *Escherichia coli* (Sigma-Aldrich, St. Louis, MO) in 0.1 mL phosphate-buffered saline (PBS).

**Preparation of Rat Prorenin HRP and Anti-HRP Antibody**

Figure 1B shows the prosegment of rat prorenin. To cover the handle region (position 11–15), we designed a decapeptide, NH2-RILLKK-MPSV-COOH, as an HRP of rat prorenin and purified it by high-pressure liquid chromatography (HPLC) on a C-18 reversed-phase column, as previously described. The purity and retention time of HPLC was 97.6% and 26.2 minutes, respectively. The mass of the product was 1185.7 and similar to the theoretical mass value (1186.0). The specific inhibitory action of HRP against prorenin activation was recently confirmed by using recombinant rat prorenin and COS-7-transfected cells expressing rat prorenin receptor (accession number AB188298 in the DNA Databank of Japan), which was originally cloned by Nguyen et al.

An anti-HRP antibody was raised against a peptide, RILLKK-MPSVC, conjugated with keyhole limpet hemocyanin in rabbits. HRP was used for determining the titer of the antisera with an ABC-AP rabbit IgG kit (Vectorstatin; Vector, Burlingame, CA) and the ligand of an affinity column for purification of the antibody. High-titer antisera were obtained 6 weeks after the first immunization. The affinity gel was prepared by conjugation of an amine-coupled gel (BioGel 102; Bio-Rad, Tokyo, Japan) through a cysteine residue of antigen peptide as a ligand. The antibody was purified with the affinity column, and the concentration of the purified antibody (3.90 mg/mL) was calculated using an extinction coefficient of 1.35 at 1 mg/mL IgG and 280 nm.

**Treatment with HRP**

Animals were treated with 0.1-mL intraperitoneal injections of vehicle (PBS) or HRP (0.1 or 0.01 mg/kg body weight) 24 h before and immediately after the injection of LPS. The effects of HRP treatment on ocular inflammation were evaluated 24 hours after LPS injection.

**Immunohistochemistry for Total Prorenin, Prorenin Receptor, and Activated Prorenin**

Immunohistochemical experiments were performed for the rat eyes with EIU. For histopathologic evaluation, the specimen was fixed with 4% paraformaldehyde (PFA) at 4°C immediately after removal and embedded in paraffin. After 3-µm deparaffinized sections were pretreated with protease K, the sections were boiled in citrate buffer with microwaves to unmask antigenic sites, and endogenous biotin was blocked (Biotin Block-
ing System X0950; Dako, Carpinteria, CA). The sections were then immersed in 3% H2O2 in methanol, to inhibit endogenous peroxidase, and were precoated with 1% nonfat milk in PBS to block nonspecific binding. For immunohistochemical staining of total prorenin (natural and nonproteolytically activated) or prorenin receptor, the rabbit anti-rat HRP antibody (1:3200) or a goat anti-rat prorenin receptor antibody (1:100) was applied, respectively, to the sections as the primary antibody. The anti-prorenin receptor antibody was raised by using the previously established COS-7 cells producing rat prorenin receptor protein. The sections were incubated with a biotin-conjugated anti-rabbit IgG or biotin-conjugated anti-goat IgG as the secondary antibody. For immunohistochemical staining of activated prorenin, a goat polyclonal antibody against the active center of renin (1:1000), which cross-reacts with both nonproteolytically and proteolytically activated prorenin but not with natural prorenin, was used.
was applied to the sections as the primary antibody. The anti-activated prorenin antibody was kindly provided by Tadashi Inagami (Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN). The sections were incubated with a biotin-conjugated anti-goat IgG as the secondary antibody. The immunohistochemical reactions were visualized by using a Vectastain ABC Standard Kit (Vector) and 0.2 mg/mL 3,3'-diaminobenzidine tetrahydrochloride (DAB; Dojindo, Kumamoto, Japan) in 0.05 M Tris-HCl (pH 7.6) containing 0.003% H$_2$O$_2$. The sections were counterstained with hematoxylin. As a negative control for staining, the first antibodies were replaced with a nonimmune rabbit or goat IgG (Dako). The immunohistochemical experiments included 6 to 18 sections from two rat eyes with EIU for each antibody.

**Lectin Labeling of Retinal Vasculature and Adherent Leukocytes**

The leukocytes adhering to the retinal vasculature were imaged by perfusion-labeling with fluorescein-isothiocyanate (FITC)-coupled concanavalin A lectin (Con A; Vector), as described previously. After deep anesthesia, the chest cavity was opened and a 20-gauge perfusion cannula was introduced into the aorta. After injection of 20 mL of PBS to remove erythrocytes and nonadherent leukocytes, 20 mL of FITC-conjugated Con A lectin was perfused. Residual unbound Con A was removed with PBS perfusion. After the eyes were enucleated, the retinas were flattened mounted. The flatmounts were imaged with an epi fluorescence microscope (IX71; Olympus, Tokyo, Japan) and the total number of Con A-stained adherent leukocytes per retina was counted.

**Aqueous Humor Analyses**

Aqueous humor was collected by anterior chamber puncture with a 27-gauge needle at 0, 12, and 24 hours after LPS injection in vehicle- and HRP (0.1 or 0.01 mg/kg body weight)-treated EIU rats. Protein concentration was determined with a protein quantification kit (Dojindo), and absorbance was measured with a microplate reader (Bio-Rad).

**Reverse Transcription–Polymerase Chain Reaction for ICAM-1, CCL2/MCP-1, and IL-6**

Total RNA was isolated from the retina with extraction reagent (TRIzol; Invitrogen, Carlsbad, CA) and reverse-transcribed with a cDNA synthesis kit (First-Strand; GE Healthcare, Piscataway, NJ) according to the manufacturer’s protocols. PCR was performed with Taq DNA polymerase (Invitrogen, Carlsbad, CA) and reverse-transcribed with a cDNA synthesis kit (First-Strand; GE Healthcare, Piscataway, NJ) according to the manufacturer’s protocols. The primer sequences were as follows: 5'-ATG TGG CAC CAC ACC TCC TAC AAT GAG CTG CG-3' (sense) and 5'-CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC-3' (antisense) for 5'-ACT; 5'-AGG CTC AGG CCT AAG AGG AC-3' (sense) and 5'-AGG CTC CCC AGA GAG GTC TA-3' (antisense) for ICAM-1; 5'-CTG GGC CTG TTG TTC ACA GGT GC-3' (sense) and 5'-CTA CAG AAG TGC TTG AGG TGG TTG-3' (antisense) for CCL2/MCP-1; and 5'-AAA ATG TGC TCT GGT CTG GTG-3' (sense) and 5'-GTT TTG CCG AGT AGA CCT CA-3' (antisense) for IL-6.

**Enzyme-Linked Immunosorbent Assay for ICAM-1, CCL2/MCP-1, and IL-6**

The animals were killed with an overdose of anesthesia, and the eyes were immediately enucleated. The retina was carefully isolated and placed into 200 µL of lysis buffer supplemented with protease inhibitors and then sonicated. The lysate was centrifuged at 15,000 rpm for 15 minutes at 4°C. The levels of ICAM-1, CCL2/MCP-1, and IL-6 in the supernatant were determined with the rat ICAM-1 kit (R&D Systems, Minneapolis, MN), the rat CCL2/MCP-1 kit (BioSource International, Camarillo, CA), and the rat IL-6 kit (R&D Systems), according to the manufacturers’ protocols. The tissue sample concentration was calculated from a standard curve and corrected for protein concentration.

![Figure 2. Tissue localization of (A) total prorenin, (B) prorenin receptor, and (C) activated prorenin in EIU eyes. Positive staining for total prorenin, prorenin receptor, and activated prorenin on the retinal vessels (arrows).](image)

**Morphometric and Statistical Analyses**

All results are expressed as the mean ± SD. The data were processed for statistical analyses with the Mann-Whitney test. Differences were considered to be statistically significant at $P < 0.05$.

**RESULTS**

**Tissue Localization of Total Prorenin, Prorenin Receptor, and Activated Prorenin in EIU**

Immunohistochemistry for prorenin prosegment, prorenin receptor, and active center of renin was performed in the rat eyes with EIU to identify the expression of total prorenin, prorenin receptor, and activated prorenin in the retina vessels (Fig. 2).

**Effects of HRP on Retinal Leukocyte Adhesion**

The retinal adherent leukocytes were imaged by perfusion-labeling with FITC-coupled Con A. Leukocyte counts were evaluated in the posterior retina around the optic disc (Figs.
3Aa–Ac), the midperipheral retina near the equator of the globe (Figs. 3Ad–Af), and the peripheral (anterior) retina next to the ora serrata (Figs. 3Ag–Ai). Retinal adherent leukocytes were few in normal rats (Figs. 3Aa, Ad, Ag). Compared with vehicle-treated EIU retina (Figs. 3Ab, Ae, Ah), HRP administration (Figs. 3Ac, Af, Ai) led to suppression of leukocyte adhesion in the EIU retina. The total number of adherent leukocytes in EIU rats (737.0 ± 248.4 cells) was significantly (P < 0.01) higher than that in age-matched normal control animals (72.2 ± 29.3 cells). HRP-treated EIU rats showed a significant decrease in leukocyte counts (503.9 ± 186.1 cells for HRP 0.01 mg/kg, P < 0.05; 440.4 ± 154.4 cells for HRP 0.1 mg/kg, P < 0.01), compared with vehicle-treated EIU rats (737.0 ± 248.4 cells; Fig. 3B).

**Effects of HRP on Leukocyte Infiltration into the Vitreous Cavity**

We observed leukocyte infiltration into the vitreous cavity of EIU eyes in the hematoxylin–eosin stained sections at the optic disc. Leukocyte infiltration anterior to the optic disc, which markedly increased with induction of EIU, decreased with HRP treatment (Fig. 3C).

**Effects of HRP on Anterior Chamber Protein Leakage**

To evaluate the anti-inflammatory effect of HRP on anterior uveitis, we analyzed protein leakage into the aqueous humor. Protein concentration in the aqueous humor of the HRP-treated EIU rats was compared with vehicle-treated EIU rats (Fig. 4). Protein concentration of the rats treated with HRP was significantly lower than that of the vehicle-treated EIU rats at 12 hours after LPS injection, and the decrease was dose dependent (Fig. 4). At 24 hours after LPS injection, protein concentration of the rats treated with HRP (0.1 mg/kg) was significantly lower than that of the vehicle-treated EIU rats, while no significant difference was detected between the HRP (0.01 mg/kg)-treated EIU rats and vehicle-treated EIU rats (Fig. 4).
Effects of HRP on Retinal Expression of Inflammatory Mediators

To determine whether HRP affects inflammatory mediators associated with the pathogenesis of EIU, retinal mRNA and protein expressions of ICAM-1, CCL2/MCP-1, and IL-6 at 6 hours (mRNA) and 24 hours (protein) after LPS injection were analyzed via RT-PCR (Fig. 5) and ELISA (Figs. 6), respectively. Retinal mRNA expression of ICAM-1, CCL2/MCP-1, and IL-6 in vehicle-treated EIU rats was higher than in age-matched normal control animals. Systemic administration of HRP substantially reduced mRNA expression of these inflammatory mediators. Similarly, HRP treatment also significantly (P < 0.05) reduced protein levels of ICAM-1 (Fig. 6A), CCL2/MCP-1 (Fig. 6B), and IL-6 (Fig. 6C).

**DISCUSSION**

The present data are the first to demonstrate that inhibition of nonproteolytic activation of prorenin leads to the suppression of ocular inflammation including leukocyte adhesion and inflam-
tration and protein leakage. Furthermore, the molecular mechanisms involving the anti-inflammatory processes were elucidated in terms of adhesion molecules, chemokines, and cytokines. Recently, we revealed the role of the RAS in ocular inflammation by showing that several pathologic parameters in EIU were suppressed by blockade of AT1-R, a receptor for the RAS final effector, angiotensin II.12 Although proteolytic activation of prorenin is well known as the classic mechanism for upregulation of the circulatory RAS, which plays an important role in hypertension, much attention has been recently paid to the mechanism by which the tissue RAS leads to pathogenesis within target organs.27,28

The present study first shows the tissue localization of prorenin receptor, together with total and activated prorenin in retinal vessels (Fig. 2). Prorenin is known to be produced in various organs including the kidney, brain, testis, ovary, and vascular endothelium. Also in the eye, prorenin was found to be present in the human surgical samples29,30 and in the rodent retina.51,52 Vitreous aspirates from patients with proliferative diabetic retinopathy contained the increased levels of prorenin.50 In the normally developing retina,52 consistent with our data from the EIU retina (Fig. 2A) and the adult retina (data not shown), prorenin is detected immunohistochemically in the retinal vessels. In contrast, prorenin receptor, recently identified and characterized17,21,22, was shown to be produced in the eye (Fig. 2B), whereas it had already been found in the heart, brain, placenta, liver, pancreas, and kidney.72

Furthermore, the present study shows that HRP, a decoy peptide for prorenin receptor, suppresses EIU-related ocular inflammation including leukocyte adhesion to the retinal vessels (Figs. 3A, 3B), leukocyte infiltration into the vitreous (Fig. 3C), and protein leakage into the anterior chamber (Fig. 4) in a time- and dose-dependent manner. These findings provide the first evidence that nonproteolytic activation of prorenin in the RAS plays a significant role in ocular inflammation.

Leukocyte adhesion to the vessel walls is an important process of inflammation. When leukocytes are recruited to inflammatory sites, adhesion molecules play essential roles in the first step of inflammation. ICAM-1 and its counterreceptor β2 (CD18) integrins (i.e., LFA-1 and Mac-1) regulate leukocyte-endothelial interaction in the pathogenesis of EIU.7,8 During the development of EIU, ICAM-1 is upregulated and expressed on vascular endothelial cells of the iris and the ciliary body shortly after LPS injection.8 In addition, several studies demonstrate that treatment with anti-ICAM-1 antibodies significantly inhibited the development of EIU.8,9 In the present study, retinal ICAM-1 upregulation in EIU was suppressed after treatment with HRP (Figs. 5, 6A). This is likely to result from the suppression of the RAS, activated in EIU with AT1-R upregulation,1,3 after HRP-induced inhibition of nonproteolytic activation of prorenin. Recent in vivo and in vitro data12,15,55 showed that angiotensin II signaling induces ICAM-1 expression via AT1-R. Collectively, the suppression of retinal inflammation observed in our study depends in part on HRP-induced inhibition of ICAM-1 via RAS downregulation. Our recent data14 showed that HRP administration results in the inhibition of nephropathy in rats with streptozotocin-induced diabetes, whereas ICAM-1 deficiency is also protective against diabetic nephropathy in db/db mice.54

Besides ICAM-1, various chemical mediators are involved in the pathogenesis of EIU. In the present study, HRP treatment led to the suppression of EIU-induced, inflammation-related molecules, including ICAM-1, IL-6, and CCL2/MCP-1. Proinflammatory effects of angiotensin II are attributable to its induction of these inflammation-related molecules, most of which are downstream products of nuclear factor (NF)-κB, a transcription factor that promotes the gene expression of various inflammatory cytokines.59 LPS-induced inflammation is mediated by the activation of NF-κB.35 Indeed, ocular inflammation is suppressed by administration of an NF-κB inhibitor in EIU.36 All evidence taken together, anti-inflammatory effects of HRP are likely to result from suppressed gene expression of NF-κB-induced molecules. These previous findings, in accordance with our data, suggest that HRP affects not only ICAM-1-mediated leukocyte adhesion but also various inflammatory processes.

In the present study, although leukocyte adhesion was substantially suppressed with HRP (0.01 mg/kg) 24 hours after LPS injection (Fig. 3B), little or no significant change was detected in protein leakage when the same dose of HRP was used at the same time point (Fig. 4). Similar discrepancy between cell adhesion and infiltration and protein leakage was also noted in several EIU studies by using neutralizing antibodies against ICAM-1,14 LFA-1,8,9 E-selectin,37 P-selectin,37 and IL-10.38 Considering that prosstagnol E2, an inflammatory mediator in addition to the adhesion molecules, is operative for protein leakage59 and that combined inhibition of both L- and P-selectin suppresses protein leakage,11,37 the cell-protein discrepancy observed in the present and previous studies is most likely attributable to differential mechanisms controlling the multiple inflammatory phases.

Although our recent report12 showed that the RAS is associated with ocular inflammation in the EIU model, the present study is the first to reveal the more detailed mechanism of nonproteolytic activation of prorenin which plays a significant role in RAS-mediated ocular inflammation. Clinically, ocular inflammation such as chronic endogenous uveitis is treated mainly with topical and/or systemic application of corticosteroids. During long-term treatment with corticosteroids, however, care must be taken to guard against both ocular and systemic complications including cataract, glaucoma, diabetes, hypertension, and osteoporosis. Therefore, the establishment of additive anti-inflammatory approaches is desirable to decrease the rate and the degree of corticosteroid-induced complications. Targeting nonproteolytically activated prorenin may prove to be useful as a novel therapeutic strategy for ocular inflammation.

References


