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

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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 immunohistochemistry. 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.[1–3] 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.[12] 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, nonproteolytic 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-
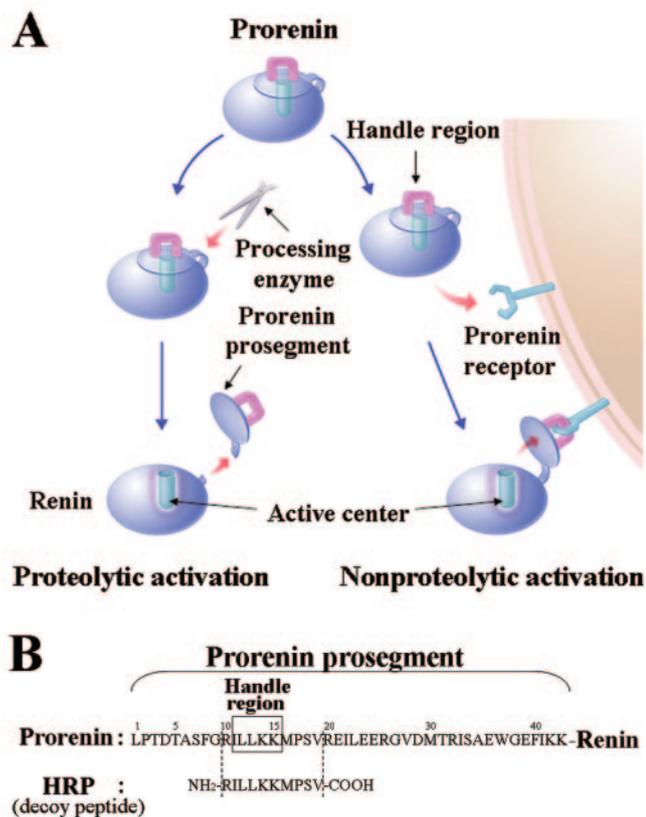
FIGURE 1. (A) Proteolytic activation of prorenin by processing enzymes versus nonproteolytic activation by prorenin receptor binding to the handle region of the prorenin prosegment. (B) Preparation of the decoy peptide corresponding to the HRP. Amino acid sequences of the rat prorenin prosegment and HRP.

matic bioactivity of renin without cleavage of the prorenin prosegment or change in molecular weight. In vitro nonproteolytic activation of prorenin was originally observed under acidic pH or low-temperature,[18–20] which are called acid-activation and cryo-activation, respectively. However, these experimental phenomena have never been found in vivo.

Our recent report[21] indicated the importance of interaction of prorenin receptor with the handle region of the prorenin prosegment for in vivo nonproteolytic activation of prorenin (Fig. 1A) and revealed the association of nonproteolytic activation with pathogenesis in the kidney. When rats with streptozotocin-induced diabetes received a peptide with the structure of the handle region of the prorenin prosegment (handle-region peptide [HRP]; Fig. 1B) as a decoy for the prorenin receptor, it potently suppressed the progression of diabetic nephropathy by inhibiting the nonproteolytic activation of prorenin and the subsequent upregulation of the RAS in the kidney. We have suggested the importance of this novel receptor-associated prorenin (RAP) system as an organ-specific RAS enhancer in disease, because the RAS, independently of soluble processing enzymes, is locally upregulated by nonsoluble, membrane-bound prorenin receptor in target organs.

Little is known, however, about the expression of prorenin receptor in the eye. It is also unclear whether the RAP system is associated with ocular disease. Herein, we report for the first time, using the rodent model of EIU which we have recently shown to be mediated by the RAS, that nonproteolytically activated prorenin plays a significant role in ocular inflammation and that HRP as a decoy for prorenin receptor is anti-inflammatory in the eye.

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),[17] 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.[21] 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),[21] which was originally cloned by Nguyen et al.[22]

An anti-rat 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 antiserum with an ABC-AP rabbit IgG kit (Vectastatin; Vector, Burlingame, CA) and as 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) the day 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 proteinase K, the sections were boiled in citrate buffer with microwaves to unmask antigenic sites, and endogenous biotin was blocked (Biotin Blocking System X0590; Dako, Carpinteria, CA). The sections were then immersed in 3% H_2O_2 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.[21] 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,[23–25]

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_2O_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 con-canavalin A lectin (Con A; Vector), as described previously.[26] 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 flatmounted. The flatmounts were imaged with an epifluorescence 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 (Toyobo, Tokyo, Japan) in a thermal controller (MiniCycler; MJ Research, Watertown, MA). The primer sequences were as follows: 5'-ATG TGG CAC CAC ACC TTC TAC AAT GAG CTG CG-3' (sense) and 5'-CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC-3' (antisense) for β-actin; 5'-AGC CTC AGG CCT AAG AGG AC-3' (sense) and 5'-AGG GGT CCC AGA GAG GTC TA-3' (antisense) for ICAM-1; 5'-CTG GGC CTG TTG TTC ACA GTT GC-3' (sense) and 5'-CTA CAG AAG TGC TTG AGG TGG TTG-3' (antisense) for CCL2/MCP-1; and 5'-AAA ATC TGC TCT GGT CTT CTG G-3' (sense) and 5'-GGT 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.
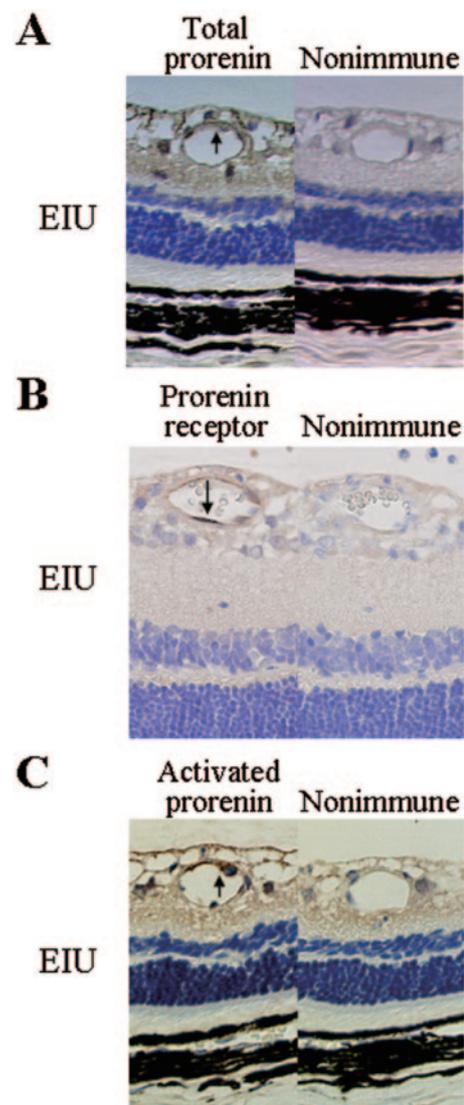

FIGURE 2. Tissue localization of (**A**) total prorenin, (**B**) prorenin receptor, and (**C**) activated prorenin in EIU eyes. Positive staining for total prorenin, prorenin receptor, and activated prorenin on the retinal vessels (*arrows*).

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, respectively. Retinal vessels were positive for total prorenin, prorenin receptor, and activated prorenin (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.
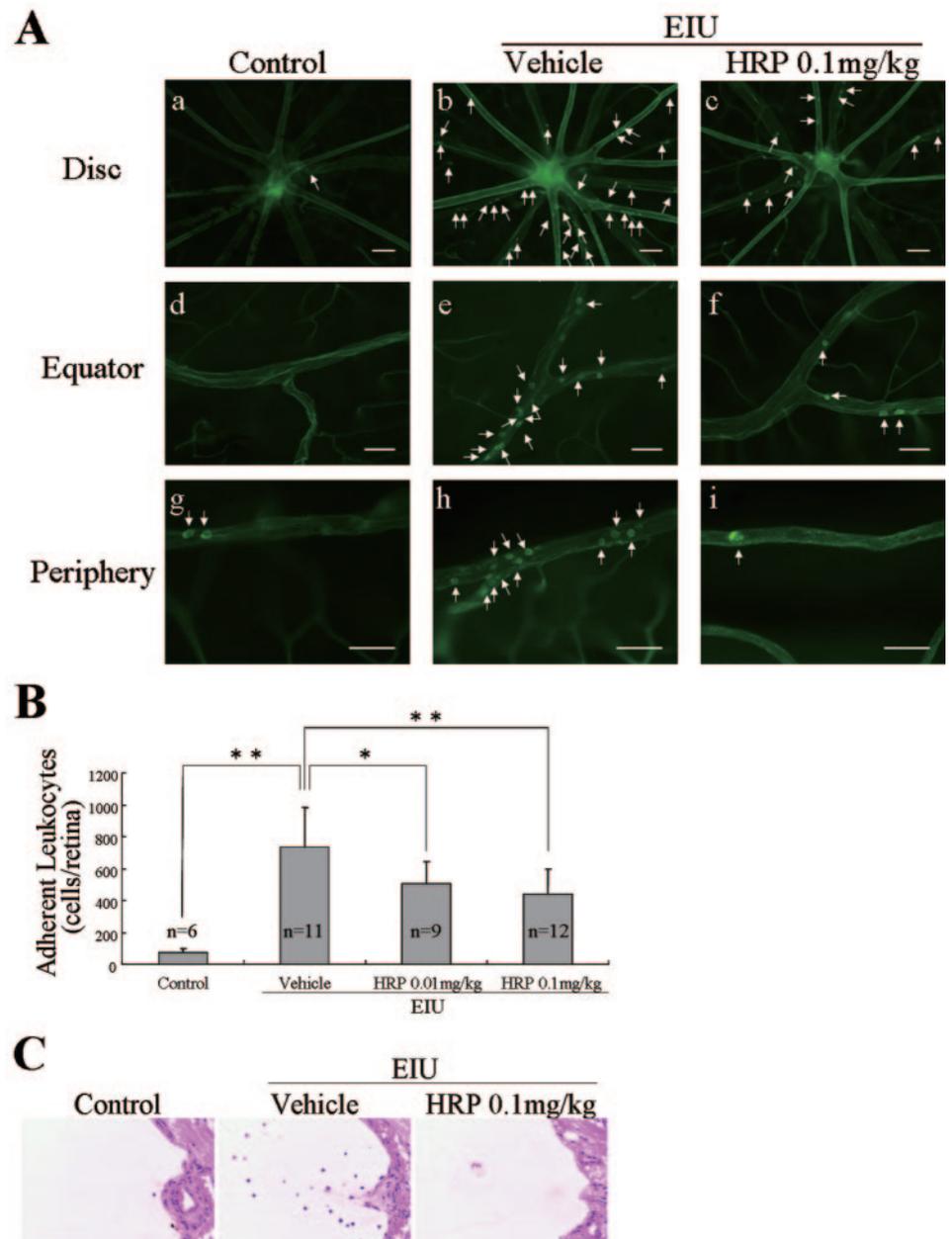
FIGURE 3. Effects of HRP on leukocyte adhesion and infiltration. (**A**) Flatmounted retinas from normal rats (**Aa**, **Ad**, **Ag**), vehicle-treated EIU rats (**Ab**, **Ae**, **Ah**), and HRP-treated EIU rats (**Ac**, **Af**, **Ai**). Vehicle-treated EIU rats showing increased leukocytes (*arrows*) adhering to the retinal vasculature compared with normal animals. Systemic treatment with HRP led to the suppression of leukocyte adhesion. Scale bar, 100 μm. (**B**) The number of retinal adherent leukocytes. HRP-treated EIU rats showed significantly fewer adherent leukocytes than did vehicle-treated animals. The results represent the mean $\pm$ SD; *$P < 0.05$, **$P < 0.01$ (Mann-Whitney test). (**C**) Leukocyte infiltration into the vitreous cavity. The number of leukocytes around the optic disc, which increased in EIU rats compared with normal control animals, was suppressed by treatment with HRP.

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 $\pm$ 248.4 cells) was significantly ($P < 0.01$) higher than that in age-matched normal control animals (72.2 $\pm$ 29.3 cells). HRP-treated EIU rats showed a significant decrease in leukocyte counts (503.9 $\pm$ 136.1 cells for HRP 0.01 mg/kg, $P < 0.05$; 440.4 $\pm$ 154.4 cells for HRP 0.1 mg/kg, $P < 0.01$), compared with vehicle-treated EIU rats (737.0 $\pm$ 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).
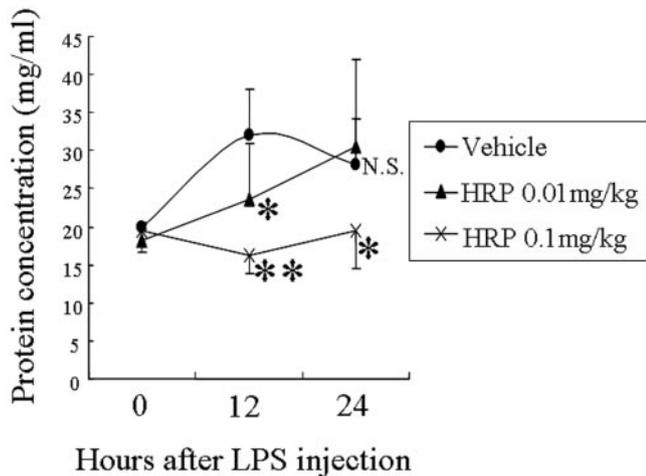
FIGURE 4. Effects of HRP on anterior uveitis. Systemic treatment with HRP resulted in dose- and time-dependent inhibition of protein leakage into the anterior chamber. The results represent the mean ± SD; $n = 6$ to 11; *$P < 0.05$, **$P < 0.01$ (Mann-Whitney test).

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).
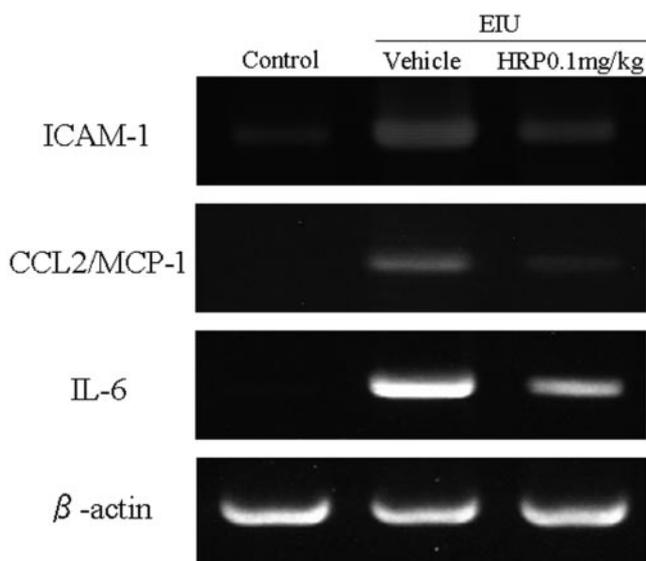

FIGURE 5. Effects of HRP on retinal expression of inflammatory mediators. Retinal mRNA expression of ICAM-1, CCL2/MCP-1, and IL-6 in vehicle-treated EIU rats increased compared with that in age-matched normal control animals, but was substantially suppressed by the administration of HRP.
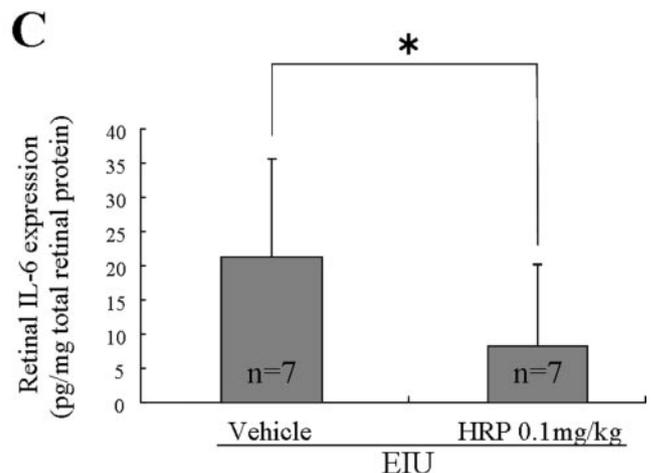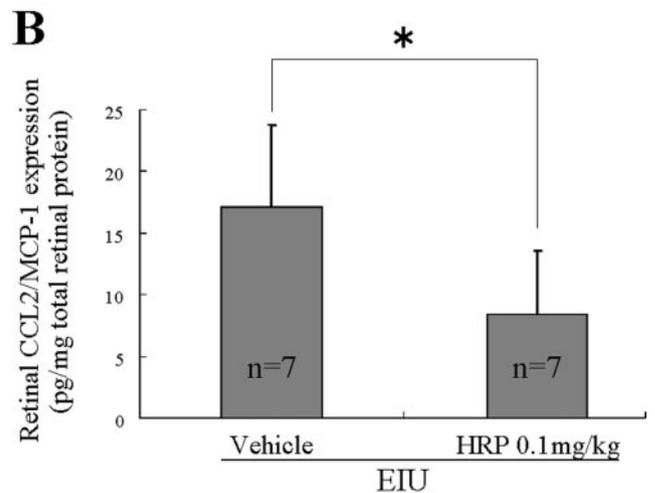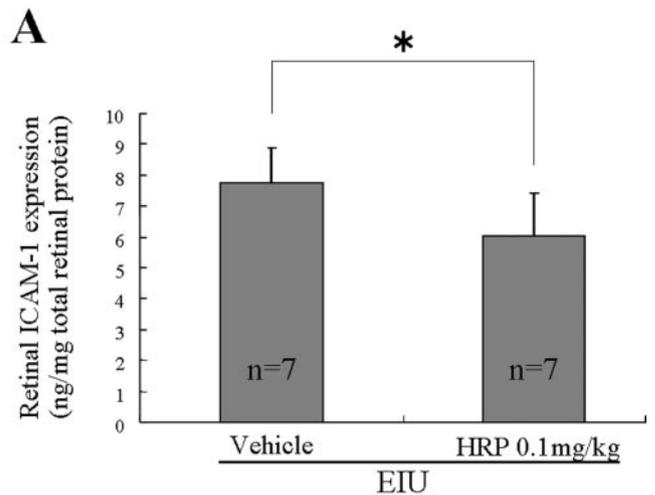

FIGURE 6. Effects of HRP on protein levels of retinal ICAM-1 (A), CCL2/MCP-1 (B), and IL-6 (C). Retinal protein levels in vehicle-treated EIU rats were significantly reduced by treatment with HRP. The results represent the mean ± SD; *$P < 0.05$ (Mann-Whitney test).

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 infil-

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 recently been 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 samples[29,30] and in the rodent retina.[31,32] Vitreous aspirates from patients with proliferative diabetic retinopathy contained the increased levels of prorenin.[30] In the normally developing retina,[32] 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 characterized,[17,21,22] 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.[22]

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.[7–9] 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,[12] after HRP-induced inhibition of nonproteolytic activation of prorenin. Recent in vivo and in vitro data[12,15,33] 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 data[18] 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.[34]

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.[35] 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,[8] LFA-1,[8,9] E-selectin,[37] P-selectin,[37] and IL-10.[38] Considering that prostaglandin E2, an inflammatory mediator in addition to the adhesion molecules, is operative for protein leakage[39] 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 report[12] 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

1. Rosenbaum JT, McDevitt HO, Guss RB, Egbert PR. Endotoxin-induced uveitis in rats as a model for human disease. *Nature.* 1980;286:611–613.
2. Bhattacherjee P, Williams RN, Eakins KE. An evaluation of ocular inflammation following the injection of bacterial endotoxin into the rat foot pad. *Invest Ophthalmol Vis Sci.* 1983;24:196–202.
3. Hoekzema R, Verhagen C, van Haren M, Kijlstra A. Endotoxin-induced uveitis in the rat: the significance of intraocular interleukin-6. *Invest Ophthalmol Vis Sci.* 1992;33:532–539.
4. Ohta K, Kikuchi T, Miyahara T, Yoshimura N. DNA microarray analysis of gene expression in iris and ciliary body of rat eyes with endotoxin-induced uveitis. *Exp Eye Res.* 2005;80:401–412.
5. 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.
6. Mo JS, Matsukawa A, Ohkawara S, Yoshinaga M. Role and regulation of IL-8 and MCP-1 in LPS-induced uveitis in rabbits. *Exp Eye Res.* 1999;68:333–340.
7. Springer TA. Signals on endothelium for lymphocyte recirculation and leukocyte emigration: the area code paradigm. *Harvey Lect.* 1993–94;89:53–103.
8. 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.
9. Whitcup SM, DeBarge LR, Rosen H, Nussenblatt RB, Chan CC. Monoclonal antibody against CD11b/CD18 inhibits endotoxin-induced uveitis. *Invest Ophthalmol Vis Sci.* 1993;34:673–681.

10. Miyamoto K, Ogura Y, Hamada M, Nishiwaki H, Hiroshiba N, Honda Y. In vivo quantification of leukocyte behavior in the retina during endotoxin-induced uveitis. *Invest Ophthalmol Vis Sci.* 1996;37:2708–2715.

11. Yamashiro K, Kiryu J, Tsujikawa A, et al. Suppressive effects of selectin inhibitor SKK-60060 on the leucocyte infiltration during endotoxin induced uveitis. *Br J Ophthalmol.* 2003;87:476–480.

12. Nagai N, Oike Y, Noda K, et al. Suppression of ocular inflammation in endotoxin-induced uveitis by blocking the angiotensin II type 1 receptor. *Invest Ophthalmol Vis Sci.* 2005;46:2925–2931.

13. Chua CC, Hamdy RC, Chua BH. Upregulation of vascular endothelial growth factor by angiotensin II in rat heart endothelial cells. *Biochim Biophys Acta.* 1998;1401:187–194.

14. Candido R, Allen TJ, Lassila M, et al. Irbesartan but not amlodipine suppresses diabetes-associated atherosclerosis. *Circulation.* 2004; 109:1536–1542.

15. Pastore L, Tessitore A, Martinotti S, et al. Angiotensin II stimulates intercellular adhesion molecule-1 (ICAM-1) expression by human vascular endothelial cells and increases soluble ICAM-1 release in vivo. *Circulation.* 1999;100:1646–1652.

16. Okamura A, Rakugi H, Ohishi M, et al. Upregulation of renin-angiotensin system during differentiation of monocytes to macrophages. *J Hypertens.* 1999;17:537–545.

17. Suzuki F, Hayakawa M, Nakagawa T, et al. Human prorenin has "gate and handle" regions for its non-proteolytic activation. *J Biol Chem.* 2003;278:22217–22222.

18. Sealey JE, Laragh JH. "Prorenin" in human plasma. *Circ Res.* 1975; 36(suppl 1):10–16.

19. Derkx FH, Schalekamp MP, Schalekamp MA. Two-step prorenin-renin conversion. Isolation of an intermediary form of activated prorenin. *J Biol Chem.* 1987;262:2472–2477.

20. Leckie BJ, McGhee NK. Reversible activation-inactivation of renin in human plasma. *Nature.* 1980;288:702–705.

21. Ichihara A, Hayashi M, Kaneshiro Y, et al. Inhibition of diabetic nephropathy by a decoy peptide corresponding to the "handle" region for nonproteolytic activation of prorenin. *J Clin Invest.* 2004;114:1128–1135.

22. Nguyen G, Delarue F, Burckle C, Bouzhir L, Giller T, Sraer JD. Pivotal role of the renin/prorenin receptor in angiotensin II production and cellular responses to renin. *J Clin Invest.* 2002;109: 1417–1427.

23. Takii Y, Figueiredo AF, Inagami T. Application of immunochemical methods to the identification and characterization of rat kidney inactive renin. *Hypertension.* 1985;7:236–243.

24. Inagami T, Murakami T, Higuchi K, Nakajo S. Roles of renal and vascular renin in spontaneous hypertension and switching of the mechanism upon nephrectomy. *Am J Hypertens.* 1991;4:15S–22S.

25. Iwai N, Inagami T, Ohmichi N, Kinoshita M. Renin is expressed in rat macrophage/monocyte cells. *Hypertension.* 1996;27:399–403.

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

27. Veniant M, Menard J, Bruneval P, Morley S, Gonzales MF, Mullins J. Vascular damage without hypertension in transgenic rats expressing prorenin exclusively in the liver. *J Clin Invest.* 1996;98: 1966–1970.

28. Peters J, Farrenkopf R, Clausmeyer S, et al. Functional significance of prorenin internalization in the rat heart. *Circ Res.* 2002;90: 1135–1141.

29. Danser AH, van den Dorpel MA, Deinum J, et al. Prorenin in vitreous and subretinal fluid of the human eye. *Clin Exp Hypertens A.* 1988;10:1297–1299.

30. Danser AH, van den Dorpel MA, Deinum J, et al. Renin, prorenin, and immunoreactive renin in vitreous fluid from eyes with and without diabetic retinopathy. *J Clin Endocrinol Metab.* 1989;68: 160–167.

31. Berka JL, Stubbs AJ, Wang DZ, et al. Renin-containing Müller cells of the retina display endocrine features. *Invest Ophthalmol Vis Sci.* 1995;36:1450–1458.

32. Sarlos S, Wilkinson-Berka JL. The renin-angiotensin system and the developing retinal vasculature. *Invest Ophthalmol Vis Sci.* 2005; 46:1069–1077.

33. Cheng ZJ, Vaskonen T, Tikkanen I, et al. Endothelial dysfunction and salt-sensitive hypertension in spontaneously diabetic Goto-Kakizaki rats. *Hypertension.* 2001;37:433–439.

34. Chow FY, Nikolic-Paterson DJ, Ozols E, Atkins RC, Tesch GH. Intercellular adhesion molecule-1 deficiency is protective against nephropathy in type 2 diabetic db/db mice. *J Am Soc Nephrol.* 2005;16:1711–1722.

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

36. Ohta K, Nakayama K, Kurokawa T, Kikuchi T, Yoshimura N. Inhibitory effects of pyrrolidine dithiocarbamate on endotoxin-induced uveitis in Lewis rats. *Invest Ophthalmol Vis Sci.* 2002;43: 744–750.

37. Suzuma I, Mandai M, Suzuma K, Ishida K, Tojo SJ, Honda Y. Contribution of E-selectin to cellular infiltration during endotoxin-induced uveitis. *Invest Ophthalmol Vis Sci.* 1998;39:1620–1630.

38. Hayashi S, Guex-Crosier Y, Delvaux A, Velu T, Roberge FG. Interleukin 10 inhibits inflammatory cells infiltration in endotoxin-induced uveitis. *Graefes Arch Clin Exp Ophthalmol.* 1996;234: 633–636.

39. Herbort CP, Okumura A, Mochizuki M. Endotoxin-induced uveitis in the rat: a study of the role of inflammation mediators. *Graefes Arch Clin Exp Ophthalmol.* 1988;226:553–558.