Role of Nonproteolytically Activated Prorenin in Pathologic, but Not Physiologic, Retinal Neovascularization

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PURPOSE. Recently, it was revealed that the inhibition of nonproteolytic activation of prorenin led to significant suppression of ocular inflammation in endotoxin-induced uveitis. The purpose of the present study was to investigate whether nonproteolytically activated prorenin plays a role in ischemia-induced retinal neovascularization.

METHODS. C57BL/6 neonatal mice were reared in an 80% concentration of oxygen from postnatal (P) day 7 to P12, followed by room-air breathing to P17 to induce ischemia-initiated retinal neovascularization. Tissue localization of activated prorenin and prorenin receptor was examined by immunohistochemistry. Animals received intraperitoneal injections of handle-region peptide (HRP), a decoy peptide corresponding to the handle region of prorenin, which inhibits prorenin receptor–mediated upregulation of the renin-angiotensin system (RAS). A concanavalin A lectin perfusion–labeling technique was used to evaluate the areas of physiologic and pathologic retinal new vessels and the number of leukocytes adhering to the vasculature. Retinal mRNA expression and protein levels of intercellular adhesion molecule (ICAM)-1, vascular endothelial growth factor (VEGF), VEGF receptor (VEGFR)-1, and VEGFR-2 were examined by RT-PCR and ELISA.

RESULTS. Retinal vessels in ischemic retinopathy eyes were positive for activated prorenin and prorenin receptor. Pathologic, but not physiologic, retinal neovascularization was significantly attenuated in HRP-treated mice compared with vehicle- or control peptide-treated animals. The number of adherent leukocytes was also significantly reduced. Retinal mRNA expression and protein levels of ICAM-1, VEGF, VEGFR-1, and VEGFR-2 in ischemic retinopathy were also significantly suppressed by the application of HRP.

CONCLUSIONS. The present findings suggest that nonproteolytic activation of prorenin selectively promotes pathologic, but not physiologic, retinal neovascularization through the inflammatory processes related to pathologic neovascularization. (*Invest Ophthalmol Vis Sci.* 2007;48:422–429) DOI:10.1167/iovs.06-0534

Retinal neovascularization is a hallmark of vision-threatening retinal diseases, including diabetic retinopathy and retinopathy of prematurity, which are major causes of blindness in adults and children, respectively. There are two distinctly different types of retinal neovascularization, physiologic and pathologic, each of which is induced by retinal ischemia. In the former, new vessels grow systematically in the retina to compensate for retinal ischemia, whereas in the latter, retinal new vessels ectopically invade the transparent vitreous, which originally lacks vasculature. Because simultaneous prevention of both types of retinal neovascularization causes retinal ischemia to be untreated, ophthalmologists await the establishment of new therapy that selectively targets pathologic neovascularization while it spares compensatory physiologic neovascularization. We have recently highlighted the molecular and cellular mechanisms differentiating pathologic from physiologic retinal neovascularization.[1–3] The influx of inflammatory cells at the growing tip of new vessels is likely to be a critical step in changing the direction of retinal neovascularization from intraretinal to extraretinal growth.

Recently, we demonstrated that angiotensin II type 1 receptor (AT1-R) blockers, widely and safely used for antihypertension therapy, have an inhibitory effect on retinal neovascularization in the murine model of ischemic retinopathy.[4] 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 condition, including inflammation and neovascularization, through the interaction of angiotensin II with AT1-R.[5–8] The initial step for upregulation of the RAS is classically known as proteolytic activation, whereby prorenin is converted to an active (mature) form of renin by 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 (ACE) 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,[9] has attracted growing attention as a local activator 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 enzymatic 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,[10–12] which are called acid-activation and cryo-

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activation, respectively. However, these experimental phenomena have never been found in vivo.

Our recent report[13] 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 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. In stroke-prone, spontaneously hypertensive rats, cardiac fibrosis developed and the RAS was activated.[14] We showed that systemic HRP administration led to significant suppression of cardiac fibrosis, suggesting the involvement of nonproteolytic activation of prorenin with the pathogenesis in the heart.[14] We suggested the importance of this novel receptor-associated prorenin (RAP) system as an organ-specific RAS enhancer in disease because membrane-bound prorenin receptor locally activated the RAS in target organs independently of diffusable processing enzymes.
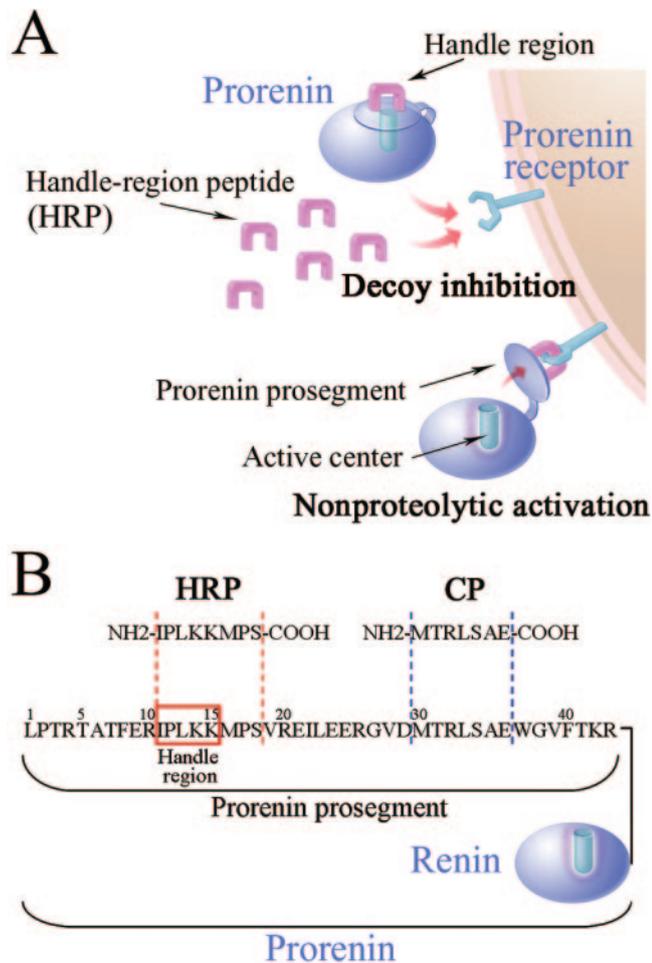

FIGURE 1. **(A)** Nonproteolytic activation of prorenin by prorenin receptor binding to the handle region, resulting in the exposure of the active center of prorenin because of its conformational change. This nonproteolytic activation is blocked by a decoy peptide, HRP, which competitively binds to prorenin receptor. **(B)** Preparation of the decoy peptide corresponding to the handle-region structure and a control peptide (CP). Amino acid sequences of the mouse prorenin prosegment, HRP and CP.

Recently, we demonstrated the expression of prorenin receptor in retinal vessels in rats with endotoxin-induced uveitis[15] and the association of the RAP system with ocular inflammation. Here we report for the first time, using the rodent model of ischemic retinopathy we recently showed to be mediated by the RAS,[4] that nonproteolytically activated prorenin plays a significant role in retinal neovascularization and that HRP as a decoy for prorenin receptor is antiangiogenic in the eye.

METHODS

Animals and Induction of Ischemic Retinopathy

C57BL/6 mice (CLEA, Tokyo, Japan) and Long-Evans rats (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 conducted 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. Postnatal day (P) 7 mice with their nursing mothers were maintained for 5 full days in 80% oxygen to generate the nonvascular retinal area, as described previously.[16] On P12, they were placed in normoxia for 5 additional days to induce retinal neovascularization. As for the rat model of ischemic retinopathy, P0 rats with their nursing mothers were maintained for 10 full days in 80% oxygen to generate the nonvascular retinal area, as described previously.[1] On P10, they were placed in normoxia for 7 additional days to induce retinal neovascularization.

Preparation of Mouse Prorenin HRP and CP

Figure 1B shows the prosegment of mouse prorenin. To cover the handle region (positions 11–15),[9] we designed a decapeptide NH2-IPLKKMPS-COOH as an HRP of mouse prorenin and purified it by high-pressure liquid chromatography (HPLC) on a C-18 reverse-phase column. The purity and retention time of HPLC were 97.4% and 6.8 minutes, respectively. The mass of the product was 913.4, similar to the theoretical mass value (913.2). The specific inhibitory action of HRP against nonproteolytic activation of prorenin in mice was confirmed in our recent in vivo data.[17] As a negative control for HRP, we also prepared a control peptide (CP), NH_2-MTRLSAE-COOH, which corresponded to positions 30 to 36 of the prorenin prosegment.

Intraperitoneal Injection of HRP and CP

After 80% oxygen exposure, pups received 0.1-mL intraperitoneal injections of vehicle (phosphate-buffered saline [PBS]), CP (1.0 mg/kg), or HRP (1.0 mg/kg) for 5 days in normoxia (21% oxygen) after hyperoxic exposure (P12-P16). The degree of retinal neovascularization and the number of adherent leukocytes were evaluated on P17.

Immunohistochemistry for Activated Prorenin and Prorenin Receptor

Immunohistochemical experiments were performed for rat eyes with ischemic retinopathy on P17. For histopathologic evaluation, the specimen was fixed with 4% paraformaldehyde (PFA) at 4°C immediately after removal and was 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). 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 activated prorenin, a goat polyclonal antibody against the active center of renin (1:1000), which cross-reacts with nonproteolytically and proteolytically activated prorenin but not with

natural prorenin,[18-20] 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). For immunohistochemical staining of prorenin receptor, a goat anti–rat prorenin receptor antibody (1: 100) was applied 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.[13] Sections were incubated with biotin-conjugated anti–goat IgG as the secondary antibody. Immunohistochemical reactions were visualized by using a standard kit (Vectastain ABC; Vector, Burlingame, CA) and (3,3'-diaminobenzidine tetrahydrochloride (DAB [0.2 mg/mL]; Dojindo Laboratories, 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 goat IgG (R&D Systems, Minneapolis, MN).

Lectin Labeling of Retinal Vasculature and Adherent Leukocytes

The retinal vasculature and adherent leukocytes were imaged by perfusion labeling with fluorescein-isothiocyanate (FITC)–coupled concanavalin A lectin (Con A; Vector), as described previously.[1] After deep anesthesia, the chest cavity was opened, and a 27-gauge cannula was introduced into the left ventricle. After injection of 2 mL PBS to remove erythrocytes and nonadherent leukocytes, 2 mL FITC-conjugated Con A was perfused. Residual unbound Con A was removed with PBS perfusion. After the eyes were enucleated, the retinas were flat mounted. Flat mounts were imaged with an epifluorescence microscope (IX71; Olympus, Tokyo, Japan), and the total number of Con A–stained adherent leukocytes per retina was counted.

RT-PCR for ICAM-1, VEGF, VEGFR-1, and VEGFR-2

Total RNA was isolated from the retina using extraction reagent (Isogen; Nippon Gene, Toyama, Japan) and reverse-transcribed with a cDNA synthesis kit (First-Strand; Pharmacia Biotech, Uppsala, Sweden) according to the manufacturers' protocols. PCR was performed using *Taq* DNA polymerase (Toyobo, Tokyo, Japan) in a thermal controller (MiniCycler; MJ Research, Watertown, MA). 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 and 5'-ATG AAC TTT CTG CTC TCT TGG-3' (sense) and 5'-TCA CCG CCT TGG CTT GTC ACA-3' (antisense) for mouse VEGF. Mouse/rat ICAM-1 PCR, human/mouse VEGF R1 PCR, and human/mouse VEGF R2 PCR (all Primer Pair; R&D Systems) were used for ICAM-1, VEGFR-1, and VEGFR-2, respectively.

ELISA for ICAM-1, VEGF, VEGFR-1, and VEGFR-2

Animals were killed with an overdose of anesthesia, and eyes were immediately enucleated. Each retina was carefully isolated and placed in 200 μL lysis buffer (0.02 M HEPES, 10% glycerol, 10 mM $Na_4P_2O_7$, 100 μM Na_3VO_4, 1% Triton, 100 mM NaF, and 4 mM EDTA [pH 8.0]) supplemented with protease inhibitors and sonicated. The lysate was centrifuged at 20,000*g* for 15 minutes at 4°C, and the ICAM-1, VEGF, VEGFR-1, and VEGFR-2 levels in the supernatant were determined with the mouse ICAM-1, VEGF, VEGFR-1, and VEGFR-2 kits (R&D Systems) according to the manufacturer's protocol. Tissue sample concentration was calculated from a standard curve and corrected for protein concentration.

Morphometric and Statistical Analyses

All results are expressed as mean ± SD. The number of leukocytes in each flat mount was counted independently by two investigators under the epifluorescence microscope. Area ratios of pathologic and physiologic neovascularization to the flat-mounted retina were measured and calculated. The morphology of the pathologic neovascularization was readily discerned from the intraretinal extension of physiologic

vessels. Values were processed for statistical analyses (Mann-Whitney *U* test). Differences were considered statistically significant at $P < 0.05$.

RESULTS

Tissue Localization of Activated Prorenin and Prorenin Receptor in Ischemic Retinopathy

Immunohistochemistry for activated prorenin and prorenin receptor was performed in rat eyes with ischemic retinopathy. Retinal vessels in ischemic retinopathy eyes were positive for
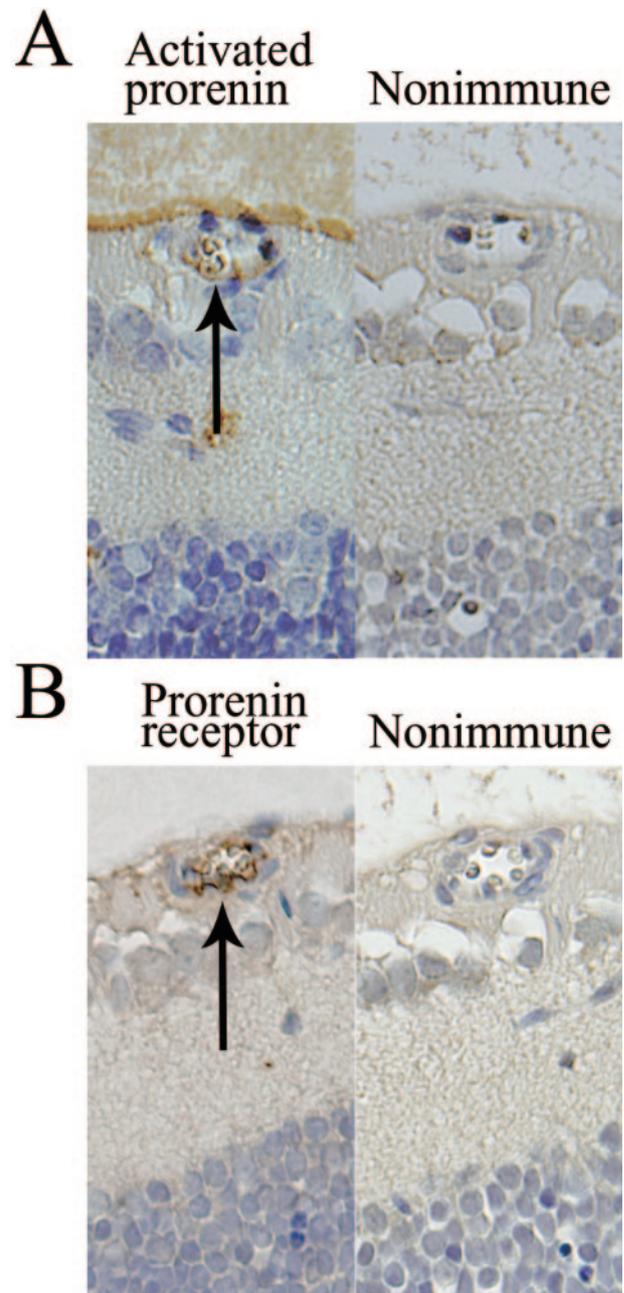

FIGURE 2. Immunohistochemical staining for activated prorenin and prorenin receptor in ischemic retinopathy eyes (**A**, **B**). Retinal vessels were positive for activated prorenin and prorenin receptor (*arrows* in **A**, **B**). Immunoreactivity was diminished in the negative control sections, where the primary antibodies were replaced with nonimmune IgG.

activated prorenin and prorenin receptor, whereas immunore-activity was diminished in negative control sections in which primary antibodies were replaced with nonimmune IgG (Figs. 2A, 2B).

Effects of HRP on Pathologic Retinal Neovascularization and Inflammation

The retinal vasculature and adherent leukocytes were imaged by perfusion labeling with FITC-coupled Con A. In healthy P17 mice, the retina was covered with physiologic (intraretinal) new vessels (Fig. 3Aa). In P17 mice with ischemic retinopathy, physiologic new vessels and pathologic neovascular buds (arrows) were both observed (Fig. 3Ab). Under higher magnification, abundant leukocytes were observed adhering to the pathologic neovascular fronds (Fig. 3B). Compared with vehicle- and CP-treated mice (Figs. 3Ab, 3Ac), systemic application of HRP led to significant suppression of pathologic neovascularization on P17 (Fig. 3Ad). The area ratio of pathologic neovascularization in the HRP-treated group was 4.8% ± 1.6%,

which was significantly ($P < 0.01$) decreased compared with the vehicle group (10.6% ± 1.5%) or the CP group (10.2% ± 0.9%; Fig. 3C). The number of adherent leukocytes in the ischemic retinopathy group (76.0 ± 27.7) was significantly ($P < 0.05$) higher than in healthy P17 mice (7.0 ± 3.9; Fig. 3E). Leukocyte counts showed a significant ($P < 0.01$) decrease in the HRP-treated group (32.1 ± 11.0) compared with the vehicle group (76.0 ± 27.7) or the CP group (70.3% ± 17.4%; Fig. 3E).

Effects of HRP on Physiologic Retinal Neovascularization

Area ratios of physiologic neovascularization were compared among healthy P17, vehicle-, CP-, and HRP-treated retinopathy groups (Figs. 3Aa-3Ad). No significant ($P > 0.05$; Fig. 3D) difference in the area of physiologic neovascularization was detected among the HRP- (66.5% ± 4.7%), vehicle- (61.9% ± 2.9%), and CP-treated (63.4% ± 2.8%) mice. To further confirm this sparing effect of HRP on physiologic vasculature, neonates
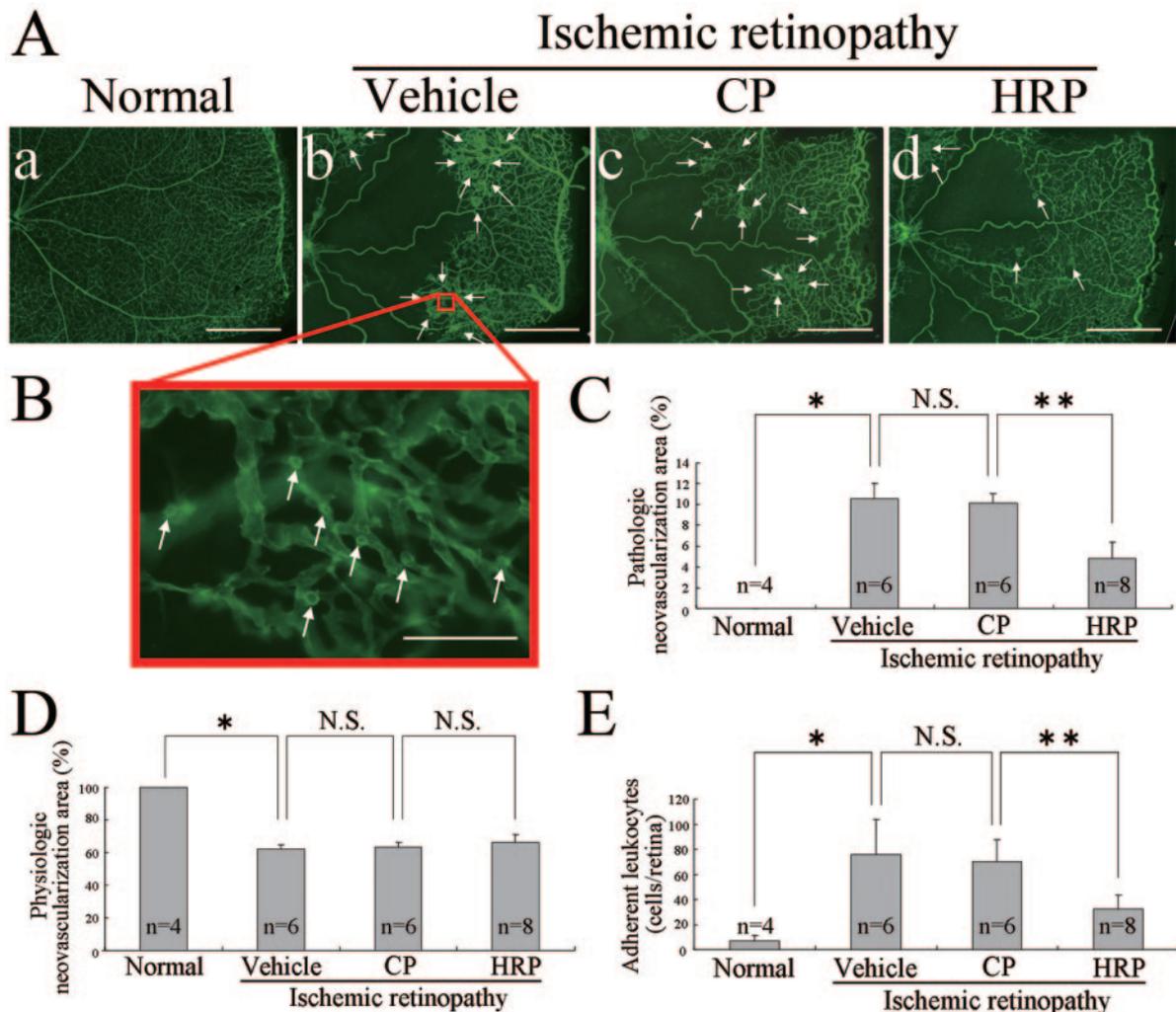

FIGURE 3. (**A**) Flat-mounted retinas from P17 healthy mice (**a**), ischemic retinopathy mice with pathologic neovascular buds (*arrows* in **b**, **c**), and HRP-treated retinopathy mice showing decreased pathologic neovascularization (*arrows* in **d**) and intact physiologic neovascularization compared with vehicle- or CP-treated animals (**b**, **c**). Scale bars: 200 μm. (**B**) Adherent leukocytes (*arrows*) accompanied by pathologic neovascularization. Scale bar: 100 μm. (**C**) Effects of HRP on pathologic neovascularization. HRP-treated retinopathy mice showing significantly less pathologic neovascularization than vehicle- or CP-treated animals. (**D**) Effects of HRP on physiologic neovascularization in ischemic retinopathy. HRP-treated mice exhibited no significant ($P > 0.05$) difference in physiologic neovascularization compared with vehicle- or CP-treated animals. (**E**) Effects of HRP on leukocyte adhesion. HRP-treated retinopathy mice showing significantly fewer adherent leukocytes than vehicle- or CP-treated mice. Results represent mean ± SD. *$P < 0.05$, **$P < 0.01$ (Mann-Whitney *U* test).
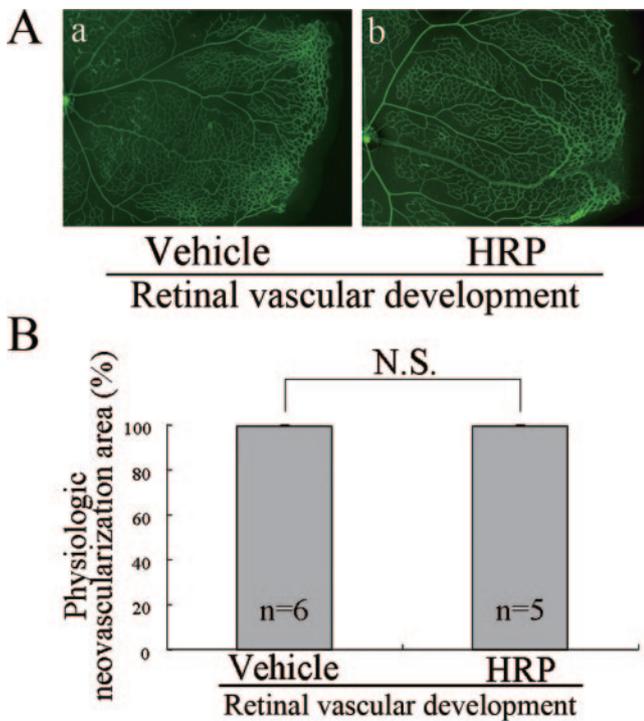
FIGURE 4. (A) Flat-mounted retinas from neonates with physiologic vascular development. No morphologic difference was detected between vehicle-treated and HRP-treated mice. (B) Effects of HRP on physiologic neovascularization during retinal development. HRP-treated mice exhibited no significant ($P > 0.05$) difference in physiologic neovascularization compared with vehicle-treated mice. Results represent mean ± SD.

undergoing retinal vascular development were used (Fig. 4A). Pups received 0.1-mL intraperitoneal injections of vehicle (PBS) or HRP (1.0 mg/kg) for 3 days in normoxia (21% oxygen) from P5 to P7 and were evaluated on P8. As for physiologic neovascularization during postnatal retinal development, no significant ($P > 0.05$; Fig. 4B) difference in physiologic neovascularization area was detected between the HRP group (99.3% ± 0.5%) and the vehicle group (99.4% ± 0.4%).

Detection of mRNA Expression and Protein Levels of ICAM-1, VEGF, VEGFR-1, and VEGFR-2

Retinas from P17 mice were subjected to RT-PCR and ELISA analyses to detect mRNA expression and protein levels of ICAM-1, VEGF, VEGFR-1, and VEGFR-2 (Figs. 5, 6). Retinal ICAM-1, VEGF, and VEGFR-1 mRNA expression and protein levels in mice with ischemic retinopathy treated with vehicle were enhanced compared with those in healthy age-matched mice. Systemic administration of HRP substantially reduced ICAM-1, VEGF, VEGFR-1, and VEGFR-2 mRNA expression (Fig. 5). Similarly, retinal ICAM-1, VEGF, VEGFR-1, and VEGFR-2 protein levels were significantly attenuated by treatment with HRP ($P < 0.01$, Figs. 6A-6D).

DISCUSSION

The present data are the first to demonstrate that the inhibition of nonproteolytic activation of prorenin leads to significant suppression of pathologic retinal neovascularization and inflammation. Furthermore, the molecular mechanisms including VEGF, VEGFR-1, VEGFR-2, and ICAM-1 were elucidated. Recently, we revealed the role of the RAS in retinal neovascularization by showing that several pathologic parameters in isch-

emic retinopathy were suppressed by blockade of AT1-R, a receptor for the RAS final effector molecule angiotensin II.[4] 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.[21,22]

The present study first shows the tissue localization of activated prorenin and prorenin receptor in retinal vessels in ischemic retinopathy (Figs. 2A-2B). Prorenin is known to be produced in various organs, including the kidney, brain, testis, ovary, and vascular endothelium. In addition, in the eye, prorenin was found to be present in surgical samples of patients[23,24] and in the rodent retina.[25,26] Vitreous aspirates from patients with proliferative diabetic retinopathy contained increased levels of prorenin.[24] In the normally developing retina,[26] consistent with our data from the ischemic retinopathy retina (Fig. 2A) and the normal P17 retina (data not shown), prorenin is detected immunohistochemically in the retinal vessels. In contrast, prorenin receptor, recently identified and characterized,[9,13,27] was shown to be produced in the retinal vessels (Fig. 2B), whereas it was already found in the heart, brain, placenta, liver, pancreas, and kidney.[27]

Furthermore, the present study shows that HRP, a decoy peptide for prorenin receptor, suppresses pathologic (Fig. 3C), but not physiologic (Figs. 3D, 4B), retinal neovascularization and leukocyte adhesion to the retinal vessels (Fig. 3E). These findings provide the first evidence that nonproteolytic activation of prorenin in the RAS plays a pivotal role in pathologic retinal neovascularization and inflammation. This is supported in part by previous reports showing that the RAS downstream inhibitors, including an ACE inhibitor and both AT1-R and AT2-R blockers, suppressed retinal neovascularization, though no mechanistic explanation was presented concerning inflammatory processes associated with pathologic neovascularization.[28,29] We have recently proposed that ischemia-induced retinal neovascularization, when it becomes pathologic, involves inflammation.[1-3] A previous immunohistochemical study pointed out the infiltration of macrophages in fibrovas-
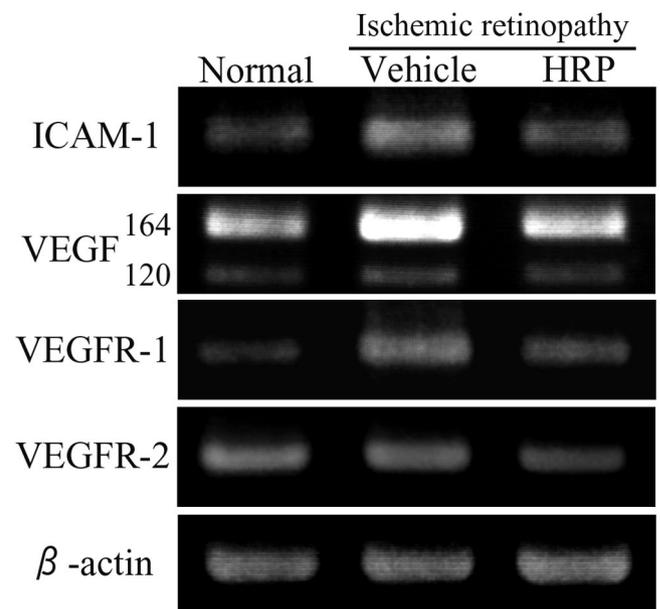

FIGURE 5. RT-PCR analyses for retinal ICAM-1, VEGF, VEGFR-1, and VEGFR-2. Retinal mRNA expression of ICAM-1, VEGF, VEGFR-1, and VEGFR-2 in ischemic retinopathy mice was substantially suppressed by treatment with HRP.
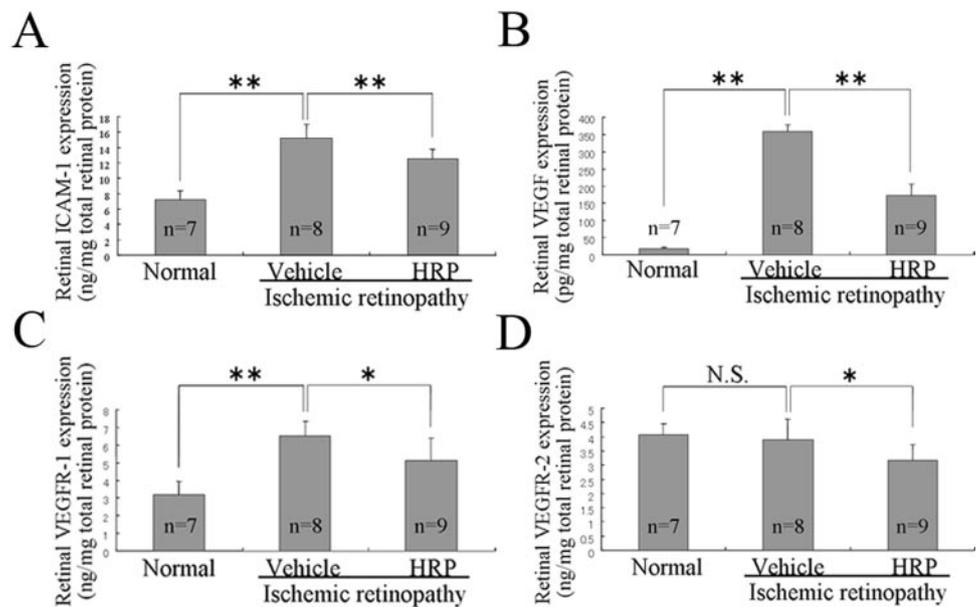
FIGURE 6. ELISA analyses for retinal ICAM-1, VEGF, VEGFR-1, and VEGFR-2. (A–D) Retinal protein levels of ICAM-1, VEGF, VEGFR-1, and VEGFR-2 were significantly lower in the HRP-treated retinopathy group than in the vehicle-treated retinopathy group. Results represent mean ± SD. *$P < 0.05$, **$P < 0.01$ (Mann-Whitney U test).

cular tissues excised at vitrectomy for proliferative diabetic retinopathy,[30] indicating a possible link between retinal neovascularization and inflammation. In an animal model of ischemic retinopathy, pathologic, but not physiologic, neovascularization was shown to be preceded and accompanied by the adhesion of inflammatory monocytes to the retinal vasculature.[1] When clodronate-liposome, a reagent that induces apoptosis specifically to monocyte/macrophage-lineage cells, was used, pathologic retinal neovascularization was suppressed without any substantial effect on physiologic neovascularization.[1] In addition, other reports have suggested the proangiogenic role of inflammatory monocytes and macrophages in murine ischemic retinopathy. Intravitreally infiltrating macrophages adjacent to the pathologic new vessels express and produce VEGF in the animal model.[31] Neutralizing antibodies against monocyte chemotactic protein (MCP)-1 and macrophage inflammatory protein (MIP)-1α were shown to reduce pathologic retinal neovascularization and inflammation.[32] Therefore, inflammatory monocytes are likely to disrupt the direction of physiologic neovascularization, triggering pathologic retinal neovascularization.

ICAM-1 is a ligand for β2-integrins constitutively expressed on the leukocyte surface and is a key adhesion molecule that controls leukocyte adhesion to the vessel walls.[33] ICAM-1, constitutively expressed on vascular endothelial cells at a low level, is swiftly upregulated during inflammation, resulting in the enhancement of leukocyte-endothelial interaction. A previous study in donor eyes demonstrated that diabetic retinas had elevated levels of ICAM-1 immunoreactivity in the vessels and the increased number of infiltrating leukocytes compared with normal retinas.[34] In a rodent model of diabetes, ICAM-1–dependent leukocyte adhesion is enhanced in the early stage,[35,36] and various retinal pathologic conditions related to long-term diabetes have been shown to be mediated by ICAM-1.[37] In vitro, Ang II was shown to induce the expression of ICAM-1 on vascular endothelial cells and to promote leukocyte–endothelial adhesion.[7] In accordance with in vitro data, HRP-treated animals in our study exhibited decreased retinal ICAM-1 mRNA expression and production and suppressed leukocyte adhesion to the retinal vessels, reasonably resulting in the inhibition of inflammation-related pathologic neovascularization.

VEGF has two cognate receptors, VEGFR-1 and VEGFR-2.[38–41] VEGF-mediated endothelial cell mitogenic activity was shown to depend not on VEGFR-1 but on VEGFR-2.[41,42] VEGFR-2 blockade in the retinopathy model was reported to suppress pathologic and physiologic neovascularization,[43] suggesting a major role of the VEGF–VEGFR-2 system in retinal neovascularization. Angiotensin II induced the in vitro expression of VEGF and VEGFR-2 mRNA on cultured bovine retinal vascular cells, enhancing VEGF-induced angiogenic activity.[44,45] In the present study, HRP application to mice with ischemic retinopathy caused substantial (52%) and modest (18%) decreases in retinal production of VEGF and VEGFR-2, respectively (Figs. 6B, 6D). Accordingly, the inhibition of pathologic neovascularization is likely attributed to the HRP-induced suppression of VEGF signaling. Interestingly, HRP administration led to selective suppression of VEGF165 (Fig. 5), the pathologic isoform capable of inducing inflammation-related pathologic neovascularization in ischemic retinopathy.[1,46] The reduced protein level of residual VEGF after HRP treatment, still higher than the physiologic level (Fig. 6B), was reasonably thought to be sufficient for promoting physiologic neovascularization.

The present data show that inhibition of nonproteolytic activation led to the significant decrease in retinal VEGFR-1 levels in ischemic retinopathy. Shih et al.[47] showed that VEGFR-1 signaling activated by placenta growth factor (PlGF)-1 led to suppression of hyperoxia-induced vaso-obliteration and suggested the possibility of VEGFR-1–mediated prevention of pathologic retinal neovascularization secondary to the decreased extent of retinal ischemia. They also described that PlGF-activated signaling of VEGFR-1 did not affect any of three types of vasoproliferation (i.e., physiologic neovascularization during normal retinal development, physiologic neovascularization after hyperoxia-induced ischemia, or pathologic neovascularization after hyperoxia-induced ischemia). In the present study, we applied HRP to mice with retinopathy during the proliferative stage after the phase of hyperoxia-induced vaso-obliteration. Our administration of HRP did not affect the extent of avascular area formation in the retinopathy mice. Given that vasoproliferation after the ischemic phase depends not on VEGFR-1[47] but on VEGFR-2,[43] VEGFR-1 downregulation on vascular endothelial cells is thought to have little or no effect on retinal neovascularization. In contrast, VEGFR-1 is well known to be expressed on inflammatory leukocytes, including monocytes.[46,48,49] The HRP-induced decrease in retinal VEGFR-1 seen in the present study, therefore, is compatible

with and explained at least in part by the suppression of VEGFR-1–bearing inflammatory leukocytes adherent to the retinal vasculature.

Hypertension is an important risk factor for the progression of diabetic retinopathy.[50–52] Strict blood pressure control with an ACE inhibitor for hypertensive patients with diabetic retinopathy significantly suppresses the progression of retinopathy,[53] indicating a possible role for circulatory RAS in ocular pathogenesis. However, diabetic retinopathy is well known to progress in normotensive patients. Treatment with an ACE inhibitor for normotensive patients with diabetic retinopathy also resulted in significant suppression of progression to proliferative retinopathy,[54] suggesting the contribution of the tissue RAS and the circulatory RAS in the pathogenesis of retinal neovascularization. Our recent report showed that HRP administration to streptozotocin-induced diabetes inhibited the development of diabetic nephropathy through suppression of the tissue RAS in the kidney without affecting the circulatory RAS.[13] In addition, diabetic patients with proliferative retinopathy had high concentrations of prorenin in the eye.[24] Nonproteolytic activation of prorenin is suggested to play an important role in the regulation of tissue RAS in the eye with retinal neovascular diseases. Targeting nonproteolytically activated prorenin may prove to be useful as a novel therapeutic strategy for vision-threatening proliferative retinopathies.

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