(Pro)renin Receptor–Mediated Signal Transduction and Tissue Renin-Angiotensin System Contribute to Diabetes-Induced Retinal Inflammation

Shingo Satofuka,[1,2] Atsuhiro Ichihara,[3] Norihiro Nagai,[1,2] Kousuke Noda,[1,2] Yoko Ozawa,[1,2] Akiyoshi Fukamizu,[4] Kazuo Tsubota,[2] Hiroshi Itoh,[3] Yuichi Oike,[5] and Susumu Ishida[1,2,6]

OBJECTIVE—The term "receptor-associated prorenin system" (RAPS) refers to the pathogenic mechanisms whereby prorenin binding to its receptor dually activates the tissue renin-angiotensin system (RAS) and RAS-independent intracellular signaling via the receptor. The aim of the present study was to define the association of the RAPS with diabetes-induced retinal inflammation.

RESEARCH DESIGN AND METHODS—Long-Evans rats, C57BL/6 mice, and angiotensin II type 1 receptor (AT1-R)-deficient mice with streptozotocin-induced diabetes were treated with (pro)renin receptor blocker (PRRB). Retinal mRNA expression of prorenin and the (pro)renin receptor was examined by quantitative RT-PCR. Leukocyte adhesion to the retinal vasculature was evaluated with a concanavalin A lectin perfusion–labeling technique. Retinal protein levels of vascular endothelial growth factor (VEGF) and intercellular adhesion molecule (ICAM)-1 were examined by ELISA. Retinal extracellular signal–regulated kinase (ERK) activation was analyzed by Western blotting.

RESULTS—Induction of diabetes led to significant increase in retinal expression of prorenin but not the (pro)renin receptor. Retinal adherent leukocytes were significantly suppressed with PRRB. Administration of PRRB inhibited diabetes-induced retinal expression of VEGF and ICAM-1. To clarify the role of signal transduction via the (pro)renin receptor in the diabetic retina, we used AT1-R–deficient mice in which the RAS was deactivated. Retinal adherent leukocytes in AT1-R–deficient diabetic mice were significantly suppressed with PRRB. PRRB suppressed the activation of ERK and the production of VEGF, but not ICAM-1, in AT1-R–deficient diabetic mice.

CONCLUSIONS—These results indicate a significant contribution of the RAPS to the pathogenesis of diabetes-induced retinal inflammation, suggesting the possibility of the (pro)renin receptor as a novel molecular target for the treatment of diabetic retinopathy. ***Diabetes*** **58:1625–1633, 2009**

From the [1]Laboratory of Retinal Cell Biology, Keio University School of Medicine, Tokyo, Japan; the [2]Department of Ophthalmology, Keio University School of Medicine, Tokyo, Japan; the [3]Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan; the [4]Center for Tsukuba Advanced Research Alliance, University of Tsukuba, Tsukuba, Japan; the [5]Department of Molecular Genetics, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan; and the [6]Inaida Endowed Department of Anti-Aging Ophthalmology, Keio University School of Medicine, Tokyo, Japan.

Corresponding author: Susumu Ishida, ishidasu@sc.itc.keio.ac.jp.

Received 21 February 2008 and accepted 27 March 2009.

Published ahead of print at http://diabetes.diabetesjournals.org on 23 April 2009. DOI: 10.2337/db08-0254.

© 2009 by the American Diabetes Association. Readers may use this article as long as the work is properly cited, the use is educational and not for profit, and the work is not altered. See http://creativecommons.org/licenses/by-nc-nd/3.0/ for details.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

See accompanying commentary, p. 1485.

Because the renin-angiotensin system (RAS) plays an important role in the regulation of systemic blood pressure, RAS inhibitors including angiotensin II type 1 receptor (AT1-R) blockers and ACE inhibitors are safely and widely used in patients with hypertension. In addition to strict control of blood glucose levels, tight blood pressure control with RAS inhibition has been shown to prevent the progression of diabetic retinopathy in the UK Prospective Diabetes Study (UKPDS) (1); however, diabetic patients are generally characterized by low renin and high prorenin levels in the plasma, indicating that the circulatory RAS is suppressed in diabetes (2). Importantly, ACE inhibition resulted in significant suppression of the progression of retinopathy in normotensive subjects with type 1 diabetes (3), suggesting that the tissue RAS in the diabetic retina is activated independently of the circulatory RAS, although the precise mechanism for activating the tissue RAS remains unclear.

The (pro)renin receptor, a recently identified transmembrane protein consisting of 350 amino acids, interacts with prorenin to exert renin activity through the conformational change of the prorenin molecule instead of the conventional proteolysis of the prorenin prosegment basically achieved by processing enzymes such as cathepsin B. Given that the membrane-bound (pro)renin receptor is reported to exist in the major organs but not in the circulation (4), the nonproteolytic activation of prorenin is hypothesized to play a critical role in the activation of the tissue, but not the circulatory, RAS. In addition, prorenin binding to its receptor is shown to cause RAS-independent signal transduction via phosphorylation of extracellular signal–regulated kinase (ERK)1/2 in cells bearing the (pro)renin receptor (4–7). Thus, we proposed the nomenclature receptor-associated prorenin system (RAPS) for the dual activation of the tissue RAS and RAS-independent signaling pathway. In streptozotocin-induced diabetes, blockade of prorenin binding to its receptor led to complete suppression of proteinuria, glomerulosclerosis, and renal production of angiotensin I and II without affecting the circulatory RAS, indicating a critical contribution of the RAPS to the pathogenesis of diabetic nephropathy (6,8–10).

Recently, we have reported that AT1-R is expressed in the fibrovascular tissues surgically excised from human eyes with proliferative diabetic retinopathy (11) and that the tissue RAS is activated in the retina of mice with streptozotocin-induced diabetes (12). Diabetic retinopathy proved to be an inflammatory disorder depending on vascular endothelial growth factor (VEGF) and intercellular adhesion molecule (ICAM)-1 (13–17). We have shown

that diabetes-induced upregulation of these inflammatory and angiogenic molecules is mediated by AT1-R signaling and required for diabetes-induced retinal leukocyte adhesion (12), indicating the association of the tissue RAS with diabetic retinopathy. This is supported by several recent reports showing that the RAS contributes to various pathological vascular conditions including inflammation and neovascularization via AT1-R signaling (18–21). However, the role of the (pro)renin receptor as a trigger to activate the tissue RAS in diabetes-induced retinal inflammation has not been defined. Although we have further shown that the tissue RAS for promoting retinal inflammation (22) and neovascularization (23) is activated by nonproteolytically processed prorenin, it has not been determined whether (pro)renin receptor–mediated intracellular signaling, the other pathway of the RAPS, is pathogenic in the eye. In the present article, we report the first evidence of a significant relationship between the RAPS and diabetes-induced retinal inflammation together with underlying molecular and cellular mechanisms.

RESEARCH DESIGN AND METHODS

Animals and induction of diabetes. Long-Evans rats (SLC, Shizuoka, Japan), C57BL/6 mice (CLEA, Tokyo, Japan), and AT1-R–deficient mice (24) (based on the C57BL/6J strain and donated by Tanabe Seiyaku, Osaka, Japan) aged 6–8 weeks were used. The mutant mice undergo targeted replacement of AT1a-R (one of the two isoforms AT1a-R and AT1b-R in rodents) loci by *lacZ* gene (24). All animal experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Animals received an intraperitoneal injection of 80 mg/kg body wt streptozotocin (Sigma, St. Louis, MO) once for rats and for 3 consecutive days for mice. Animals with plasma glucose levels >250 mg/dl 7 days after injection were considered diabetic.

Preparation of (pro)renin receptor blockers. To cover the handle region (positions 11–15) of the prorenin molecule, which is the binding site of the (pro)renin receptor (25), we designed decoy peptides NH_2-RILLKKMPSV-COOH (6,9,10,22,26) and NH_2-IPLKKMPS-COOH (8,23) as rat and mouse (pro)renin receptor blockers (PRRBs), respectively, and purified them by high-performance liquid chromatography on a C-18 reverse-phase column as previously described. We applied rat PRRB to diabetic rats (Figs. 1 and 2) and mouse PRRB to diabetic mice (Figs. 3–7). The specific inhibitory action of PRRB against prorenin binding with the (pro)renin receptor and subsequent ERK activation has been confirmed in our present (supplemental Figs. 1 and 2, available in an online appendix at http://diabetes.diabetesjournals.org/cgi/content/full/db08-0254/DC1) and recent (27,28) in vitro data. The specific inhibitory action of PRRB against the tissue RAS or RAPS in vivo was also confirmed in our recent data (6,8–10,22,23,26). As a negative control, rat PRRB was inactivated by heat denaturation at 100°C for 10 min and used as control peptide. We also generated a scramble peptide NH_2-KPMLISKP-COOH for mouse PRRB as another negative control.

Treatment with PRRB, control peptide, scramble peptide, or the AT1-R blocker losartan. Diabetic animals were intraperitoneally injected with vehicle (0.25% DMSO in PBS), 1.0 mg/kg control peptide, 1.0 mg/kg scramble peptide, 0.1 or 1.0 mg/kg PRRB, or 20 mg/kg of the AT1-R blocker losartan (Cayman Chemical, Ann Arbor, MI) daily for seven consecutive weeks immediately after the establishment of diabetes (7 days after streptozotocin injection) until the end of the study. Daily injection was based on our preliminary experiments showing the time course of serum concentration of PRRB injected into normal mice and measured by BIAcore 2000 (GE Healthcare Bio-Sciences KK, Tokyo, Japan) (supplemental Fig. 3). In our preliminary experiments, 20 mg/kg losartan proved to be the maximal-effect dose for inhibiting leukocyte adhesion to the retinal vessels of diabetic mice (data not shown).

Quantification of retinal adherent leukocytes. The retinal vasculature and adherent leukocytes were imaged by perfusion labeling with fluorescein isothiocyanate (FITC)-coupled concanavalin A lectin (ConA) (Vector, Burlingame, CA), as previously described (29). After deep anesthesia, the chest cavity was opened and a 20 or 27 G cannula (for rats or mice, respectively) was introduced into the left ventricle. After injection of PBS to remove erythrocytes and nonadherent leukocytes, 20 or 2 ml (for rats or mice, respectively) of FITC-conjugated ConA was perfused. After the eyes were enucleated, the retinas were flat mounted. The flat mounts were imaged using an epifluorescence microscope (IX71; Olympus, Tokyo, Japan), and the total number of ConA-stained adherent leukocytes per retina was determined.

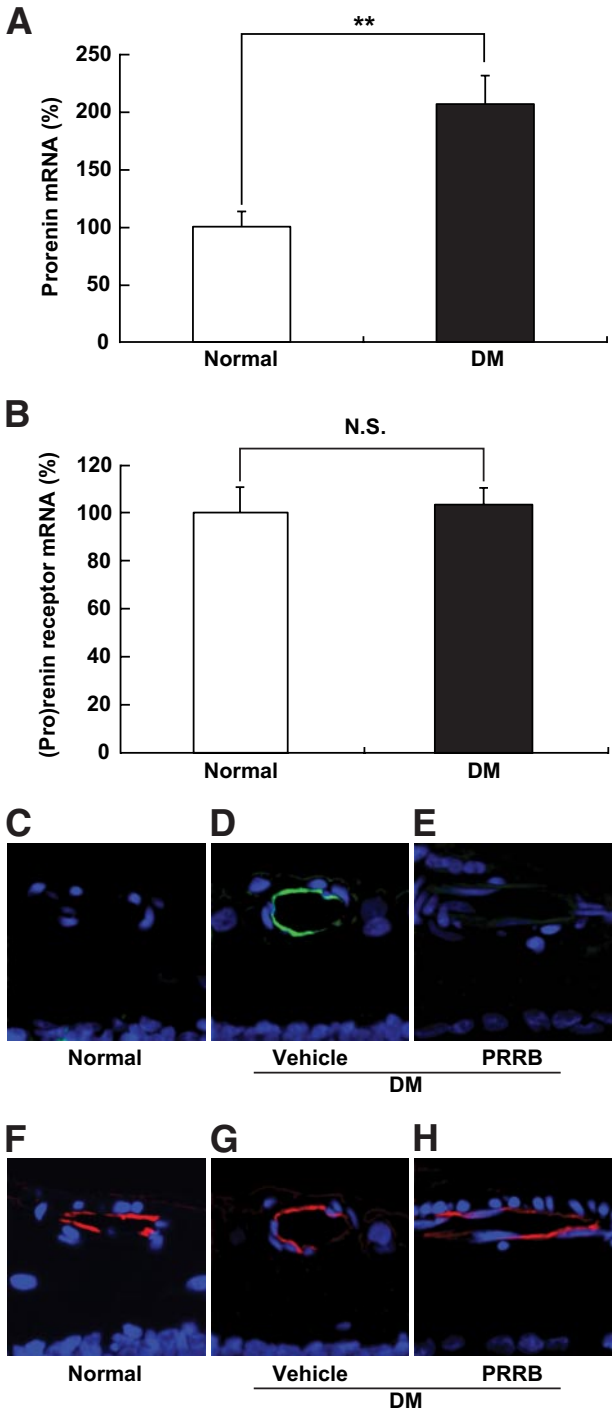

FIG. 1. Upregulation of prorenin expression and tissue localization of prorenin and the (pro)renin receptor in the diabetic retina. *A*: Upregulation of prorenin mRNA levels, analyzed by real-time RT-PCR, in the retina by inducing diabetes (DM) (n = 8). *B*: (Pro)renin receptor mRNA levels were unchanged following induction of diabetes (n = 8). Immunostaining for non–proteolytically activated prorenin (*C–E*) (green fluorescence) and the (pro)renin receptor (*F–H*) (red fluorescence) in retinal vessels. **P < 0.01. NS, nonsignificant. (A high-quality digital representation of this figure is available in the online issue.)

Quantitative RT-PCR analyses for prorenin and the (pro)renin receptor. We isolated total RNA from the retina with an RNeasy Mini Kit (Qiagen, Tokyo, Japan) and performed a real-time quantitative RT-PCR with the TaqMan One-Step RT-PCR Master Mix Reagents Kit, an ABI Prism 7700 HT

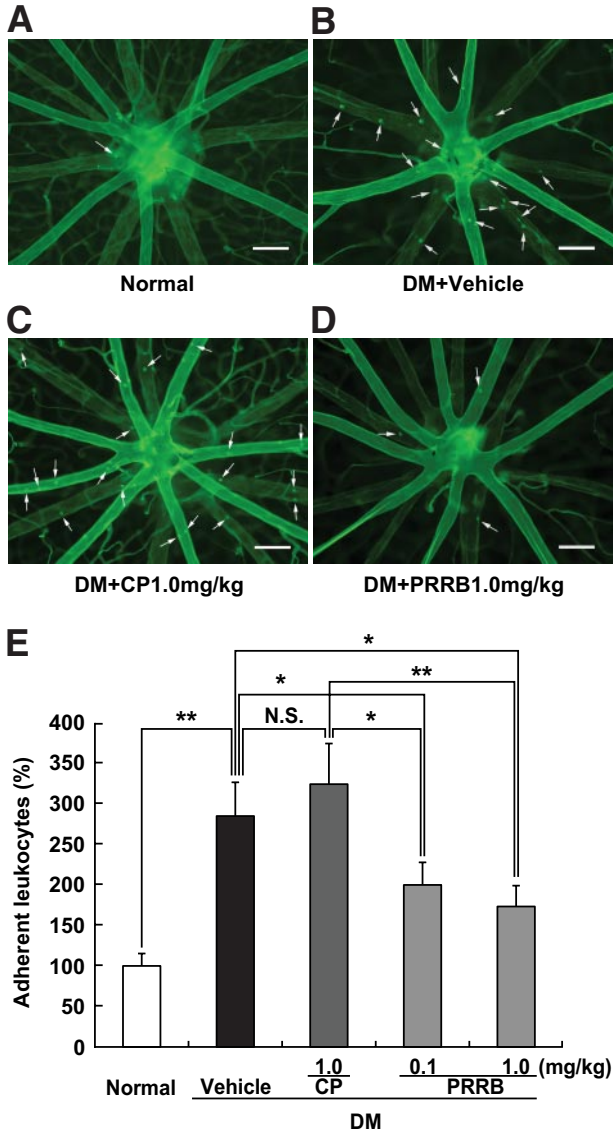

FIG. 2. Inhibitory effect of PRRB on retinal leukocyte adhesion. Flat-mounted retinas from normal (*A*) and diabetic (DM) rats treated with vehicle (*B*), control peptide (CP) (*C*), or PRRB (*D*). Scale bars: 100 μm. *E*: The number of retinal adherent leukocytes. PRRB-treated diabetic rats showed significantly fewer adherent leukocytes than did vehicle- or control peptide-treated diabetic rats (n = 5–13). *P < 0.05 and **P < 0.01. NS, nonsignificant. (A high-quality digital representation of this figure is available in the online issue.)

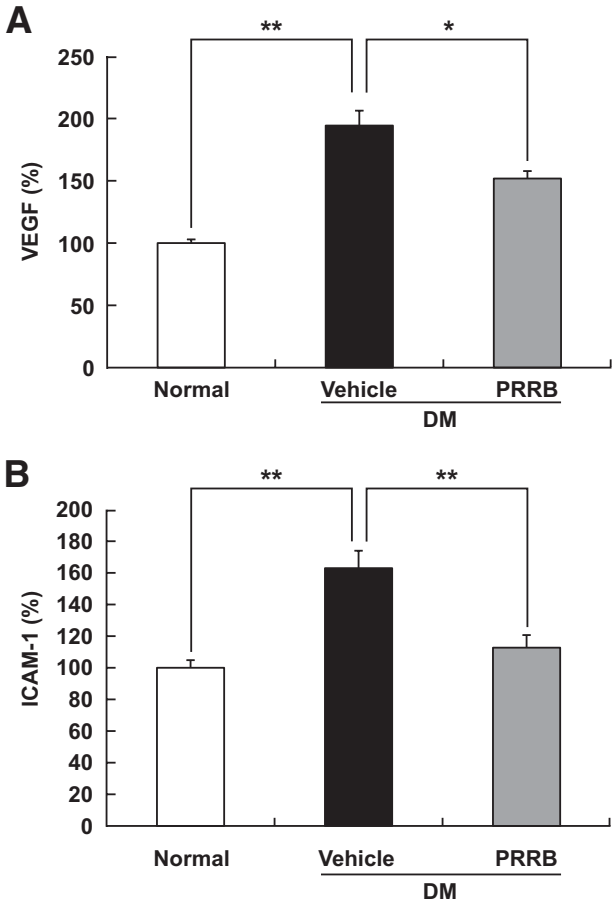

FIG. 3. Inhibitory effect of PRRB on retinal expression of inflammatory molecules. PRRB significantly suppressed retinal protein levels of VEGF (*A*) and ICAM-1 (*B*) analyzed by ELISA, both of which were elevated after induction of diabetes (DM) (n = 8–11). *P < 0.05 and **P < 0.01.

Detection System (Applied Biosystems, Foster City, CA), and probes and primers for the rat genes that encode prorenin, the (pro)renin receptor, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as previously described (9,10,26).

Immunohistochemistry for nonproteolytically activated prorenin and the (pro)renin receptor. Rat eyes were fixed with 4% paraformaldehyde and embedded in paraffin. After deparaffinized sections were precoated with 4% skim milk to block nonspecific binding, a rabbit polyclonal antibody against the gate region of rat prorenin (1:100) or a goat polyclonal antibody against the rat (pro)renin receptor (1:100) was applied as the primary antibody. An antibody against the gate region binds only to nonproteolytically activated prorenin but not to inactive prorenin or proteolytically activated prorenin (i.e., renin) (6). The anti-(pro)renin receptor antibody was raised by using the previously established COS-7 cells producing rat (pro)renin receptor protein (9). The sections were then incubated with a biotin-conjugated IgG as the secondary antibody and TOTO-3 (1:500) (Molecular Probes, Eugene, OR) for nuclear staining. The immunohistochemical reactions were visualized by using a Vectastain ABC Standard Kit (Vector Laboratories, Burlingame, CA) and Tyramide Signal Amplification Fluorescein System (PerkinElmer, Boston, MA) according to the manufacturer's protocol. The samples were viewed with the scanning laser confocal microscope.

Western blot analyses for phosphorylated and total forms of ERK1/2. The isolated retina was placed into the lysis buffer. After blocking nonspecific binding with 5% skim milk, polyvinylidene fluoride membranes were incubated with a mouse monoclonal antibody against phosphorylated ERK1/2 (1:1,000; Cell Signaling Technology, Beverly, MA), total ERK1/2 (1:1,000; Cell Signaling Technology), or α-tubulin (1:1,000; Sigma). Membranes were then incubated with biotin-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) followed by avidin-biotin complex (Vectastain ABC Elite Kit; Vector Laboratories). Finally, the signals were detected through enhanced chemiluminescence (ECL Blotting Analysis System; GE Healthcare).

Enzyme-linked immunosorbent assay for VEGF and ICAM-1. The isolated retina was placed into the lysis buffer. The protein levels of VEGF and ICAM-1 were determined with enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems). The tissue sample concentration was calculated from a standard curve and corrected for protein concentration.

RESULTS

Metabolic parameters. Animals with streptozotocin-induced diabetes showed a significant decrease in body weight ($P < 0.01$) and a significant increase in blood glucose levels ($P < 0.01$) compared with those in age-matched nondiabetic controls (Table 1). Treatment with PRRB did not significantly change these metabolic parameters ($P > 0.05$) (Table 1).

Prorenin mRNA expression was upregulated in the diabetic retina. To elucidate the involvement of prorenin and the (pro)renin receptor in the pathogenesis of diabetic

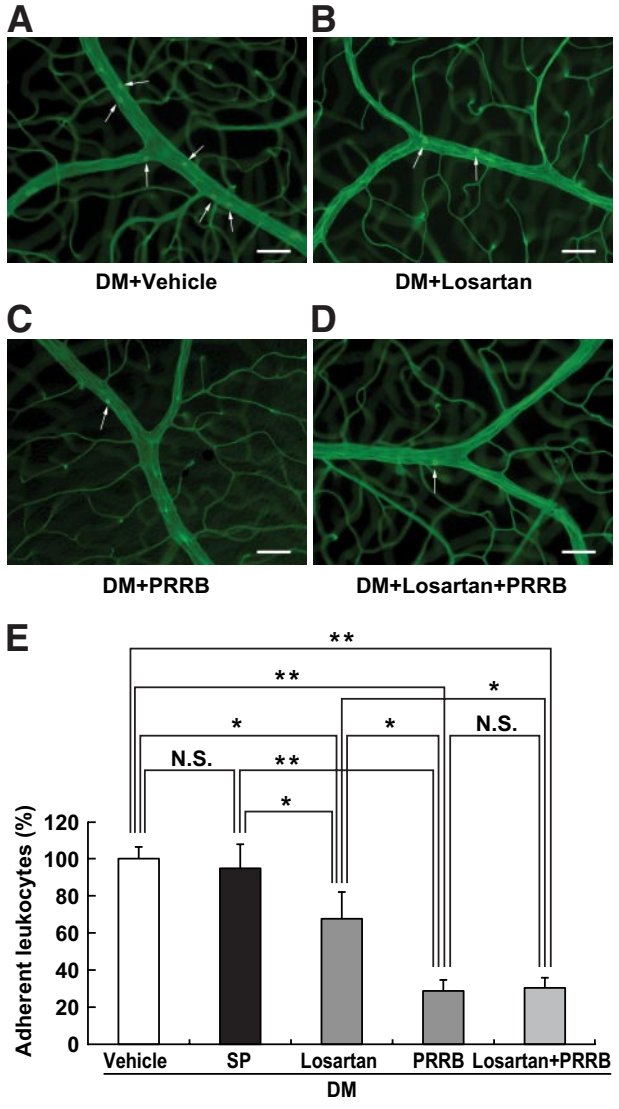

FIG. 4. PRRB was more potent in inhibiting diabetes-induced retinal leukocyte adhesion than the AT1-R blocker losartan. Flat-mounted retinas from diabetic (DM) mice treated with vehicle (*A*), losartan (*B*), PRRB (*C*), or losartan plus PRRB (*D*). Scale bars: 50 μm. *E*: Compared with losartan treatment, PRRB application led to significant ($P < 0.05$) suppression of leukocyte adhesion to the diabetic retinal vessels (n = 10–14). *$P < 0.05$. **$P < 0.01$. NS, nonsignificant; SP, scramble peptide. (A high-quality digital representation of this figure is available in the online issue.)

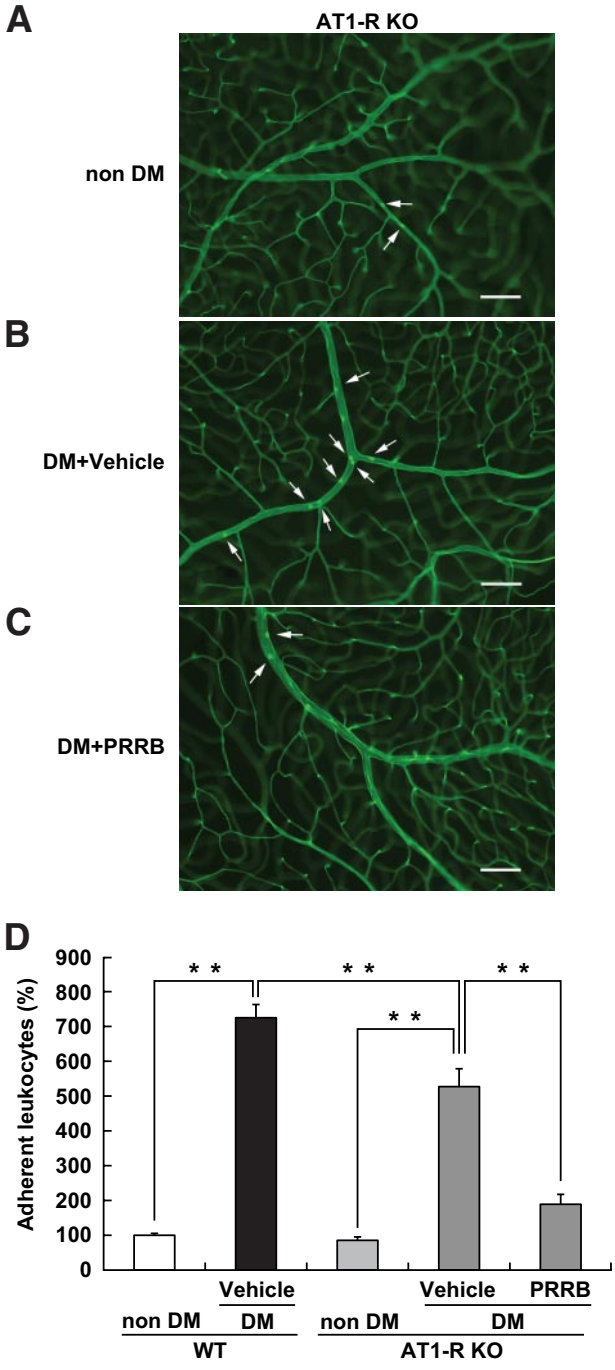

FIG. 5. RAS-independent (pro)renin receptor–mediated intracellular signaling contributes to diabetes-induced retinal leukocyte adhesion. Flat-mounted retinas from nondiabetic (*A*) and diabetic (DM) AT1-R–deficient mice treated with vehicle (*B*) or PRRB (*C*). Scale bars: 100 μm. *D*: The number of retinal adherent leukocytes. Compared with diabetic wild-type (WT) animals, diabetic AT1-R–deficient mice exhibited a significant reduction of the number of adherent leukocytes. PRRB administration to diabetic AT1-R–deficient mice showed significantly fewer adherent leukocytes than did vehicle administration (n = 6–8). **$P < 0.01$. KO, knockout. (A high-quality digital representation of this figure is available in the online issue.)

retinopathy, we first performed quantitative RT-PCR analyses for prorenin and the (pro)renin receptor in the retina. Prorenin mRNA levels (ratio to GAPDH mRNA) were upregulated ($P < 0.01$) in the diabetic retina compared with those in age-matched normal control rats (Fig. 1*A*). In contrast, mRNA levels of the (pro)renin receptor showed no significant differences ($P > 0.05$) between diabetic rats and normal controls (Fig. 1*B*).

Diabetes-induced activation of prorenin colocalized with the (pro)renin receptor was attenuated with PRRB. Immunohistochemistry for normal, vehicle-, or PRRB-treated diabetic rat eyes was performed to identify the tissue localization of nonproteolytically activated prorenin and the (pro)renin receptor. Immunoreactivity of activated prorenin in the retinal vessels was negligible in nondiabetic controls (Fig. 1*C*), substantially enhanced in diabetes (Fig. 1*D*), and abrogated by PRRB application to the background signal (Fig. 1*E*). In contrast, immunoreactivity of the (pro)renin receptor (Fig. 1*F*) was unaltered by induction of diabetes (Fig. 1*G*) or treatment with PRRB (Fig. 1*H*).

PRRB treatment suppressed diabetes-induced retinal leukocyte adhesion. The retinal adherent leukocytes were imaged by perfusion labeling with FITC-coupled

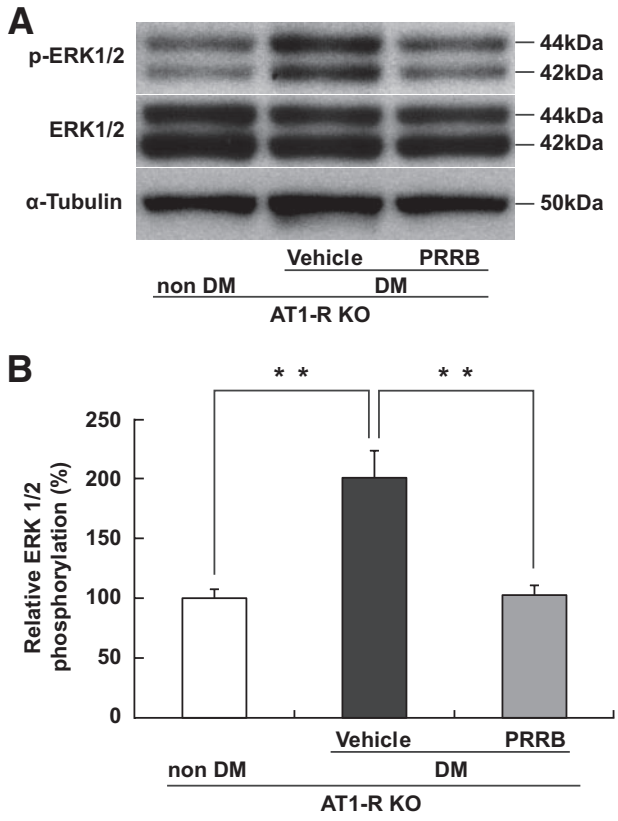

FIG. 6. RAS-independent (pro)renin receptor–mediated intracellular signaling contributes to ERK1/2 activation in the diabetic retina. *A* and *B*: Western blotting for phosphorylated and total levels of ERK1/2 in AT1-R–deficient mice with diabetes (DM). PRRB suppressed relative phosphorylation of ERK1/2 (n = 12). **P < 0.01. KO, knockout.

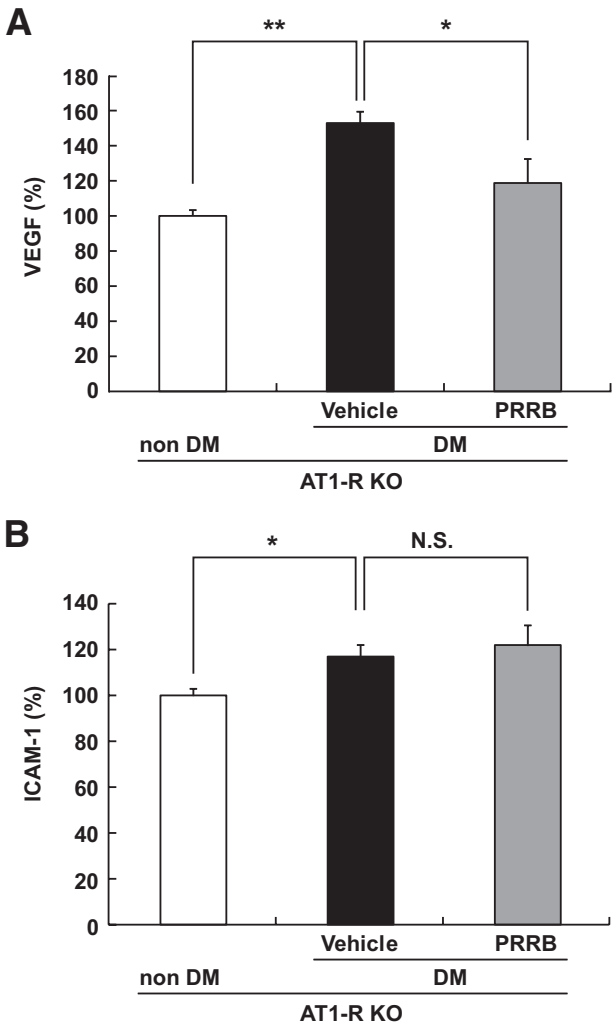

FIG. 7. RAS-independent (pro)renin receptor–mediated intracellular signaling contributes to diabetes (DM)-induced expression of VEGF but not ICAM-1. Effects by blocking intracellular signaling via the (pro)renin receptor on protein levels of diabetes-related inflammatory molecules. VEGF levels were significantly suppressed with PRRB (*A*) (n = 12–14). *B*: ICAM-1 levels showed no significant (NS) differences (n = 12–14). *P < 0.05 and **P < 0.01. KO, knockout.

ConA. Leukocyte counts were evaluated in the whole retina from the posterior retina around the optic disc (Fig. 2*A–D*) to the peripheral (anterior) retina next to the ora serrata. The total number of adherent leukocytes was significantly ($P < 0.01$ for both) higher in vehicle-treated (Fig. 2*B*) and control peptide-treated (Fig. 2*C*) diabetic rats than in normal age-matched controls (Fig. 2*A*). PRRB-treated diabetic rats (Fig. 2*D*) showed a significant decrease in the leukocyte count in a dose-dependent fashion compared with that in vehicle-treated ($P < 0.05$) or control peptide-treated ($P < 0.01$) diabetic rats (Fig. 2*E*).

PRRB treatment suppressed diabetes-induced upregulation of retinal VEGF and ICAM-1 production. Retinal protein levels of VEGF (Fig. 3*A*) and ICAM-1 (Fig. 3*B*) were significantly ($P < 0.01$) higher in vehicle-treated diabetic mice than in age-matched normal controls. PRRB significantly suppressed retinal protein levels of these inflammatory molecules known as pathogenic in the diabetic retina (Fig. 3*A* and *B*).

PRRB was more potent in inhibiting diabetes-induced retinal leukocyte adhesion than the AT1-R blocker losartan. We examined retinal leukocyte adhesion for comparison among diabetic mice treated with vehicle, scramble peptide, the AT1-R blocker losartan, PRRB, and losartan plus PRRB. Compared with vehicle (Fig. 4*A*) or scramble peptide treatment, the inhibitory effect of PRRB (71.3% inhibition) (Fig. 4*C*) on diabetic retinal inflammation was more potent than that of losartan alone (32.4% inhibition) (Fig. 4*B*) and was equivalent to that of losartan plus PRRB (Fig. 4*D*), showing that PRRB in combination with losartan caused little or no additive effect compared with PRRB alone (Fig. 4*E*). Thus, PRRB was suggested to inhibit both angiotensin II–dependent (32.4% contribution) and –independent (71.3 − 32.4 = 38.9% contribution) actions.

RAS-independent (pro)renin receptor signaling contributed to diabetes-induced retinal leukocyte adhesion. To clarify the role of RAS-independent intracellular signaling via the (pro)renin receptor, we used mice in which the RAS was deactivated by genetic ablation of AT1-R. The number of adherent leukocytes in the retinal vasculature was significantly ($P < 0.01$) higher in diabetic wild-type mice than in nondiabetic wild-type controls. Compared with diabetic wild-type animals, diabetic AT1-R–deficient mice exhibited a significant ($P < 0.01$; 27.3%) reduction of the number of adherent leukocytes. Compared with that in nondiabetic AT1-R–deficient mice (Fig. 5*A*), induction of diabetes (Fig. 5*B*) significantly ($P < 0.01$) increased the number of adherent leukocytes in the retinal vasculature. The number of retinal adherent leukocytes was significantly ($P < 0.01$; 46.6% of diabetic wild-type mice) smaller in PRRB-treated diabetic AT1-R–deficient

TABLE 1
Metabolic parameters

	Normal rats	Diabetic rats			Normal wild-type mice	Diabetic wild-type mice				Nondiabetic AT1-R knockout mice	Diabetic AT1-R knockout mice	
		Vehicle	Control peptide	PRRB		Vehicle	Scramble peptide	Losartan	PRRB	Losartan plus PRRB	Vehicle	PRRB
n	7	12	6	7	12	17	6	5	17	4	10	11
Body weight (g)	448.6 ± 13.5	280.2 ± 9.3*	271.4 ± 15.7*	289.2 ± 24.1*	27.2 ± 0.3	22.8 ± 0.4*	23.0 ± 0.4*	23.5 ± 0.5*	23.0 ± 0.4*	23.4 ± 0.4*	26.9 ± 0.4	22.6 ± 0.6*	22.9 ± 0.6*
Blood glucose (mg/dl)	139 ± 5	534 ± 22*	537 ± 39*	531 ± 33*	149 ± 6	540 ± 14*	538 ± 25*	531 ± 22*	528 ± 15*	523 ± 28*	147 ± 8	584 ± 9*	576 ± 11*

*$P < 0.01$ compared with normal or nondiabetic animals.

mice (Fig. 5*C*) at the dose of 1.0 mg/kg than in vehicle-treated diabetic AT1-R–deficient mice (Fig. 5*D*). These data with pharmacological blockade (Fig. 4) and genetic ablation (Fig. 5) of AT1-R indicated dual contribution of the tissue RAS (32.4 and 27.3%, respectively) and RAS-independent (pro)renin receptor signaling (38.9 and 46.6%) to diabetes-induced retinal inflammation.

RAS-independent (pro)renin receptor signaling contributed to diabetes-induced activation of retinal ERK1/2. To further determine whether RAS-independent intracellular signaling via the (pro)renin receptor contributes to the activation of ERK1/2 in the diabetic retina, phosphorylated ERK1/2 was examined in the retina from AT1-R–deficient diabetic mice. PRRB treatment suppressed phosphorylated but not total ERK1/2 (Fig. 6*A*). Relative phosphorylation of ERK1/2, increased by inducing diabetes, was significantly ($P < 0.01$) suppressed by PRRB application, whereas no significant ($P > 0.05$) differences were detected in total ERK1/2 protein levels (Fig. 6*B*).

RAS-independent (pro)renin receptor signaling contributed to retinal expression of VEGF but not ICAM-1. To examine whether RAS-independent intracellular signaling via the (pro)renin receptor contributes to the upregulation of the pathogenic molecules responsible for diabetes-induced retinal inflammation (Fig. 3), diabetes was induced in AT1-R–deficient mice to measure retinal levels of VEGF and ICAM-1 protein. Compared with that in nondiabetic AT1-R–deficient mice, retinal expression of VEGF ($P < 0.01$) (Fig. 7*A*) and ICAM-1 ($P < 0.05$) (Fig. 7*B*) was significantly enhanced by diabetes induction. PRRB application to AT1-R–deficient diabetic mice led to significant suppression of VEGF ($P < 0.05$) (Fig. 7*A*) but not ICAM-1 ($P > 0.05$) (Fig. 7*B*) compared with vehicle treatment in AT1-R–deficient diabetic mice.

DISCUSSION

The present study reveals for the first time, to our knowledge, several important findings concerning the role of the (pro)renin receptor in diabetes-induced retinal inflammation. First, induction of diabetes led to significant upregulation of prorenin expression in the retina, and diabetes-induced activation of prorenin colocalized with its receptor in the retinal vessels was substantially attenuated with PRRB (Fig. 1). Second, diabetes-induced leukocyte adhesion to the retinal vasculature was suppressed by PRRB treatment, indicating that prorenin binding with its receptor contributes to the pathogenesis in the diabetic retina (Fig. 2). Third, the molecular mechanisms in the suppression of retinal leukocyte adhesion proved to include the inhibitory effects of PRRB on the retinal expression of VEGF and ICAM-1, both of which are known as key factors responsible for diabetes-induced retinal inflammation (Fig. 3).

Recently, surgical sample data have revealed the elevated levels of angiotensin II in the vitreous of eyes with proliferative diabetic retinopathy (30) and diabetic macular edema (31). In accordance with the findings of these clinical laboratory studies, we have shown that induction of diabetes to mice led to a significant increase in retinal expression and production of the RAS components (12). Although these findings indicated the association of the tissue RAS with diabetic retinopathy, the precise mechanism for activating the tissue RAS in the diabetic retina has not been determined. In parallel, little has been known about the bioactivity of the prorenin molecule per se other

than its function as the inactive precursor of renin. Bilateral nephrectomy resulted in significant decline of plasma renin but not prorenin to an undetectable level, suggesting that the proteolytic processing of prorenin to renin is confined to the kidney and that other organs including the eye are capable of producing only prorenin, but not renin (32,33). Elevated prorenin levels in the plasma of long-standing diabetes (2,34,35) have indicated that prorenin may be a hallmark of microvascular complications in diabetes. Indeed, the vitreous levels of prorenin were higher in eyes with proliferative diabetic retinopathy than in those with nonproliferative retinopathy or without retinopathy (36), suggesting the involvement of prorenin with the pathogenesis of diabetic retinopathy. Reasonably, retinal mRNA levels of prorenin were elevated following induction of diabetes (Fig. 1*A*), whereas (pro)renin receptor levels were unaltered (Fig. 1*B*). The observed ligand-receptor expression change is compatible with our recent data on diabetic nephropathy (9,10) and choroidal neovascularization (37). These findings, in concert with the immunohistochemical results (Fig. 1*C–H*), suggest the significance of the ligand-based regulation in the activation of the RAPS. Importantly, nonproteolytically activated prorenin was localized in diabetic retinal vessels bearing the (pro)renin receptor (Fig. 1*D* and *G*), showing that the initiating step of the tissue RAS is prepared in the diabetic retina.

The molecular and cellular mechanisms underlying the pathogenesis of diabetic retinopathy are not fully understood; however, increasing evidence has suggested the involvement of inflammatory processes including cytokine upregulation and leukocyte infiltration, causing diabetic retinopathy to be regarded as an inflammatory disease (14,17,29,38,39). Retinal vasculature in diabetes is accompanied by inflammatory cell adhesion (40), which triggers vascular hyperpermeability (14) and pathological neovascularization (39). ICAM-1, constitutively expressed on vascular endothelial cells at a low level, is swiftly upregulated during inflammation, resulting in an enhancement of leukocyte-endothelium interaction. Previous studies using donor eyes from diabetic subjects (13) and experimentally induced diabetes (14,29) demonstrated that retinal ICAM-1 expression was elevated together with leukocyte adhesion and infiltration. Antibody-based blockade or genetic ablation of ICAM-1 led to significant suppression of vascular hyperpermeability in early diabetes (14) or capillary loss in established diabetes (38). VEGF, a potent angiogenic and proinflammatory factor, plays a central role in the pathogenesis of diabetic retinopathy. In patients with diabetic retinopathy, VEGF levels in the intraocular fluid were increased not only during the proliferative stage (15) but also during the nonproliferative stage characterized by macular edema (16). Interestingly, angiotensin II levels are elevated and correlated with VEGF levels in the vitreous fluid of patients with diabetic macular edema (31). Angiotensin II has been shown to induce ICAM-1 (41) and VEGF (42) via AT1-R in previous in vivo and in vitro studies. Also in the murine model of streptozotocin-induced diabetes, we have recently shown that the tissue RAS enhanced retinal expression of these inflammatory molecules and subsequent leukocyte adhesion to the retinal vasculature, all of which were suppressed by AT1-R blockade (12). In concert with the previous data, the currently observed PRRB-induced suppression of diabetes-induced retinal inflammation (Figs. 2 and 3) indicates that the tissue RAS is activated in the diabetic retina by (pro)renin receptor–mediated nonproteolytic activation of prorenin, leading to AT1-R signaling–mediated VEGF and ICAM-1 upregulation and retinal leukocyte adhesion.

The present study further revealed the role of RAS-independent (pro)renin receptor signaling in diabetes-induced retinal inflammation. The use of two different methods of inactivating AT1-R (Figs. 4 and 5) confirmed that PRRB functioned to inhibit both RAS-dependent and -independent mechanisms underlying retinal leukocyte adhesion. These data are the first to show the involvement of the RAPS, i.e., (pro)renin receptor–mediated signal transduction and tissue RAS activation, in the pathogenesis of diabetic retinopathy. We have recently shown the contribution of (pro)renin receptor signaling to diabetic nephropathy using AT1-R–deficient mice. AT1-R–deficient mice with streptozotocin-induced diabetes exhibited reduced proteinuria and glomerulosclerosis in the early phase compared with wild-type mice with diabetes, indicating a significant role of the tissue RAS in diabetic nephropathy. Surprisingly, these renal events in AT1-R–deficient diabetic mice later progressed to the equivalent levels seen in wild-type diabetic mice (8). The glomerulosclerosis observed in AT1-R–deficient diabetic mice was associated with ERK activation, which was completely blocked together with the phenotype by sustained application of PRRB, suggesting that the redundant pathways of the RAPS were involved in the pathogenesis of diabetic nephropathy (8). In addition, we administered PRRB to AT1-R–deficient diabetic mice and confirmed the significant role of intracellular signaling via the (pro)renin receptor in the diabetes-induced retinal leukocyte adhesion (Fig. 5). Importantly, PRRB application to AT1-R–deficient diabetic mice led to significant suppression of ERK activation, a known key intracellular signaling via the (pro)renin receptor (Fig. 6). Of diabetes-related inflammatory molecules (the expression of which was inhibited by PRRB [Fig. 3]), VEGF but not ICAM-1 was also regulated by (pro)renin receptor signaling per se (Fig. 7). The data are supported by and consistent with our recent report on choroidal neovascularization showing that (pro)renin receptor signaling selectively induces VEGF and monocyte chemoattractant protein-1 out of several inflammatory and angiogenic molecules mediated by AT1-R (37). These new findings (Figs. 4–7) clarified molecular and cellular mechanisms mediated by RAS-independent intracellular signaling via the (pro)renin receptor in diabetes-induced retinal inflammation. In addition to our recent reports showing that the RAPS contributes to glomerulosclerosis in the kidney and fibrosis in the heart (6–10,26), the present data are the first to show the association of the RAPS with inflammation in the eye (Fig. 8).

In the present data, there was a discrepancy between in vivo (Figs. 1–7 and supplemental Fig. 3) and in vitro (supplemental Figs. 1–2) doses of PRRB. In vivo, intraperitoneal injection of PRRB at a dose of 1.0 $mg \cdot kg^{-1} \cdot day^{-1}$ led to a serum concentration of $\leq$110 ng/ml ($\sim$100 nmol/l), which was cleared within 24 h (supplemental Fig. 3). Because the maximal concentration of PRRB in the serum is 50–100 times higher than the physiological level of endogenous prorenin (1–2 nmol/l), the currently used dose of 1.0 $mg \cdot kg^{-1} \cdot day^{-1}$ is thought to be sufficient to inhibit the in vivo binding of prorenin to the receptor. Indeed, a lower dose of 0.1 $mg \cdot kg^{-1} \cdot day^{-1}$ (presumably 10 nmol/l in the serum [5–10 times higher than physiological prorenin]) was also effective (Fig. 2). In vitro, however, much higher doses of PRRB (10–100 μmol/l) were required to

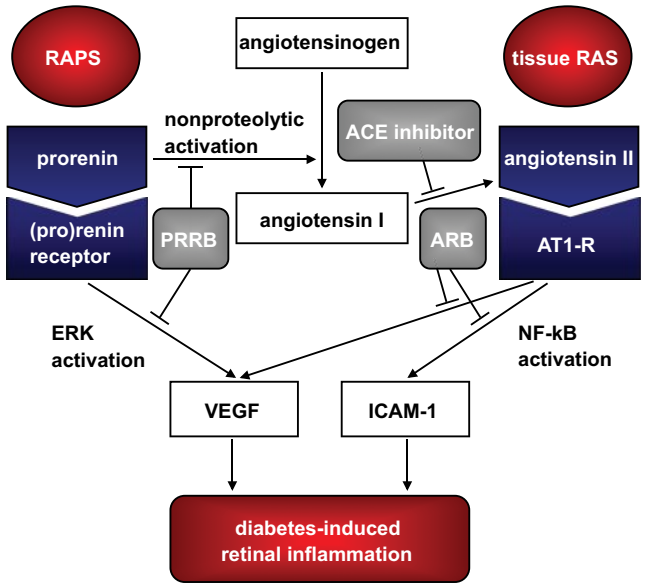

FIG. 8. A scheme showing the significant involvement of the RAPS with the pathogenesis of diabetes-induced retinal inflammation.

suppress prorenin-stimulated ERK activation (supplemental Fig. 2). This would be attributable at least in part to the in vitro property of cellular localization of the (pro)renin receptor. In cultured vascular smooth muscle cells (43), indeed, the (pro)renin receptor was shown to be localized mainly to the cytoplasm and sparsely to the cellular surface, suggesting less efficacy of PRRB exerted in vitro than in vivo.

Recently, several studies have failed to show the inhibitory effect of PRRB on in vivo (44,45) and in vitro (46,47) models. These negative data on PRRB were generated by using in vivo (44,45) models in which the tissue RAS does not originally play any role or in vitro (46,47) models applied with too low concentration ($\leq$1 μmol/l) of PRRB. The glomerulosclerosis in the clipped kidney in the rat model of 2-kidney, 1-clip hypertension has already been shown to be ischemic lesion resistant to RAS inhibition (48). The dTG rats overexpressing human renin and angiotensinogen exhibited significant increase in plasma renin and angiotensin II (activation of the circulatory but not tissue RAS), causing hypertension-induced cardiac and renal damages (45). As expected, PRRB did not alter these pathological conditions as a result of tissue RAS-independent mechanisms (44,45).

Although hypertension is a known risk factor for the progression of diabetic retinopathy (49,50), there are indeed a large number of normotensive patients with diabetic retinopathy who have the potential risk of hypotension caused by the use of antihypertensive agents including AT1-R blockers and ACE inhibitors. In contrast, because the (pro)renin receptor is present in the major organs but not in the circulation, PRRB does not affect the circulatory RAS or systemic blood pressure (9,10). Interestingly, PRRB administration to the diabetic retina was shown to cause not only tissue RAS deactivation but also additional suppression of (pro)renin receptor signaling–mediated expression of VEGF—the major pathogenic factor responsible for the development of diabetic retinopathy (Fig. 8). Collectively, inhibition of the RAPS with PRRB may prove more useful as a novel therapeutic strategy for diabetic retinopathy than RAS suppression with conventional AT1-R blockers or ACE inhibitors.

ACKNOWLEDGMENTS

This work was supported by the Japanese Ministry of Education, Culture, Sports, Science and Technology (grant-in-aid for scientific research 18791296 to S.S.).

No potential conflicts of interest relevant to this article were reported.

REFERENCES

1. UK Prospective Diabetes Study Group. Efficacy of atenolol and captopril in reducing risk of macrovascular and microvascular complications in type 2 diabetes: UKPDS 39. BMJ 1998;317:713–720
2. Luetscher JA, Kraemer FB, Wilson DM, Schwartz HC, Bryer-Ash M. Increased plasma inactive renin in diabetes mellitus. A marker of microvascular complications. N Engl J Med 1985;312:1412–1417
3. Chaturvedi N, Sjolie AK, Stephenson JM, Abrahamian H, Keipes M, Castellarin A, Rogulja-Pepeonik Z, Fuller JH. Effect of lisinopril on progression of retinopathy in normotensive people with type 1 diabetes. The EUCLID Study Group. EURODIAB Controlled Trial of Lisinopril in Insulin-Dependent Diabetes Mellitus. Lancet 1998;351:28–31
4. 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
5. Huang Y, Noble NA, Zhang J, Xu C, Border WA. Renin-stimulated TGF-beta1 expression is regulated by a mitogen-activated protein kinase in mesangial cells. Kidney Int 2007;72:45–52
6. Ichihara A, Kaneshiro Y, Takemitsu T, Sakoda M, Nakagawa T, Nishiyama A, Kawachi H, Shimizu F, Inagami T. Contribution of nonproteolytically activated prorenin in glomeruli to hypertensive renal damage. J Am Soc Nephrol 2006;17:2495–2503
7. Kaneshiro Y, Ichihara A, Sakoda M, Takemitsu T, Nabi AH, Uddin MN, Nakagawa T, Nishiyama A, Suzuki F, Inagami T, Itoh H. Slowly progressive, angiotensin II-independent glomerulosclerosis in human (pro)renin receptor-transgenic rats. J Am Soc Nephrol 2007;18:1789–1795
8. Ichihara A, Suzuki F, Nakagawa T, Kaneshiro Y, Takemitsu T, Sakoda M, Nabi AH, Nishiyama A, Sugaya T, Hayashi M, Inagami T. Prorenin receptor blockade inhibits development of glomerulosclerosis in diabetic angiotensin II type 1a receptor-deficient mice. J Am Soc Nephrol 2006;17:1950–1961
9. Ichihara A, Hayashi M, Kaneshiro Y, Suzuki F, Nakagawa T, Tada Y, Koura Y, Nishiyama A, Okada H, Uddin MN, Nabi AH, Ishida Y, Inagami T, Saruta T. 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
10. Takahashi H, Ichihara A, Kaneshiro Y, Inomata K, Sakoda M, Takemitsu T, Nishiyama A, Itoh H. Regression of nephropathy developed in diabetes by (pro)renin receptor blockade. J Am Soc Nephrol 2007;18:2054–2061
11. Nagai N, Noda K, Urano T, Kubota Y, Shinoda H, Koto T, Shinoda K, Inoue M, Shiomi T, Ikeda E, Tsubota K, Suda T, Oike Y, Ishida S. Selective suppression of pathologic, but not physiologic, retinal neovascularization by blocking the angiotensin II type 1 receptor. Invest Ophthalmol Vis Sci 2005;46:1078–1084
12. Nagai N, Izumi-Nagai K, Oike Y, Koto T, Satofuka S, Ozawa Y, Yamashiro K, Inoue M, Tsubota K, Umezawa K, Ishida S. Suppression of diabetes-induced retinal inflammation by blocking the angiotensin II type 1 receptor or its downstream nuclear factor-kappaB pathway. Invest Ophthalmol Vis Sci 2007;48:4342–4350
13. McLeod DS, Lefer DJ, Merges C, Lutty GA. Enhanced expression of intracellular adhesion molecule-1 and P-selectin in the diabetic human retina and choroid. Am J Pathol 1995;147:642–653
14. Miyamoto K, Khosrof S, Bursell SE, Rohan R, Murata T, Clermont AC, Aiello LP, Ogura Y, Adamis AP. Prevention of leukostasis and vascular leakage in streptozotocin-induced diabetic retinopathy via intercellular adhesion molecule-1 inhibition. Proc Natl Acad Sci U S A 1999;96:10836–10841
15. Aiello LP, Avery RL, Arrigg PG, Keyt BA, Jampel HD, Shah ST, Pasquale LR, Thieme H, Iwamoto MA, Park JE, Nguyen HV, Aiello LM, Ferrara, King GL. Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. N Engl J Med 1994;331:1480–1487
16. Tanaka Y, Katoh S, Hori S, Miura M, Yamashita H. Vascular endothelial growth factor in diabetic retinopathy. Lancet 1997;349:1520
17. Ishida S, Usui T, Yamashiro K, Kaji Y, Ahmed E, Carrasquillo KG, Amano S, Hida T, Oguchi Y, Adamis AP. VEGF164 is proinflammatory in the diabetic retina. Invest Ophthalmol Vis Sci 2003;44:2155–2162
18. Candido R, Allen TJ, Lassila M, Cao Z, Thallas V, Cooper ME, Jandeleit-Dahm KA. Irbesartan but not amlodipine suppresses diabetes-associated atherosclerosis. Circulation 2004;109:1536–1542

19. Tamarat R, Silvestre JS, Durie M, Levy BI. Angiotensin II angiogenic effect in vivo involves vascular endothelial growth factor- and inflammation-related pathways. Lab Invest 2002;82:747–756
20. Egami K, Murohara T, Shimada T, Sasaki K, Shintani S, Sugaya T, Ishii M, Akagi T, Ikeda H, Matsuishi T, Imaizumi T. Role of host angiotensin II type 1 receptor in tumor angiogenesis and growth. J Clin Invest 2003;112:67–75
21. Moravski CJ, Kelly DJ, Cooper ME, Gilbert RE, Bertram JF, Shahinfar S, Skinner SL, Wilkinson-Berka JL. Retinal neovascularization is prevented by blockade of the renin-angiotensin system. Hypertension 2000;36:1099–1104
22. Satofuka S, Ichihara A, Nagai N, Yamashiro K, Koto T, Shinoda H, Noda K, Ozawa Y, Inoue M, Tsubota K, Suzuki F, Oike Y, Ishida S. Suppression of ocular inflammation in endotoxin-induced uveitis by inhibiting nonproteolytic activation of prorenin. Invest Ophthalmol Vis Sci 2006;47:2686–2692
23. Satofuka S, Ichihara A, Nagai N, Koto T, Shinoda H, Noda K, Ozawa Y, Inoue M, Tsubota K, Itoh H, Oike Y, Ishida S. Role of nonproteolytically activated prorenin in pathologic, but not physiologic, retinal neovascularization. Invest Ophthalmol Vis Sci 2007;48:422–429
24. Sugaya T, Nishimatsu S, Tanimoto K, Takimoto E, Yamagishi T, Imamura K, Goto S, Imaizumi K, Hisada Y, Otsuka A, Uchida H, Sugiura M, Fukuta K, Fukamizu A, Murakami K. Angiotensin II type 1a receptor-deficient mice with hypotension and hyperreninemia. J Biol Chem 1995;270:18719–18722
25. Suzuki F, Hayakawa M, Nakagawa T, Nasir UM, Ebihara A, Iwasawa A, Ishida Y, Nakamura Y, Murakami K. Human prorenin has "gate and handle" regions for its non-proteolytic activation. J Biol Chem 2003;278:22217–22222
26. Ichihara A, Kaneshiro Y, Takemitsu T, Sakoda M, Suzuki F, Nakagawa T, Nishiyama A, Inagami T, Hayashi M. Nonproteolytic activation of prorenin contributes to development of cardiac fibrosis in genetic hypertension. Hypertension 2006;47:894–900
27. Nurun NA, Uddin NM, Nakagawa T, Iwata H, Ichihara A, Inagami T, Suzuki F. Role of "handle" region of prorenin prosegment in the non-proteolytic activation of prorenin by binding to membrane anchored (pro)renin receptor. Front Biosci 2007;12:4810–4817
28. He M, Zhang L, Shao Y, Wang X, Huang Y, Yao T, Lu L. Inhibition of renin/prorenin receptor attenuated mesangial cell proliferation and reduced associated fibrotic factor release. Eur J Pharmacol 2009;606:155–161
29. Joussen AM, Poulaki V, Qin W, Kirchhof B, Mitsiades N, Wiegand SJ, Rudge J, Yancopoulos GD, Adamis AP. Retinal vascular endothelial growth factor induces intercellular adhesion molecule-1 and endothelial nitric oxide synthase expression and initiates early diabetic retinal leukocyte adhesion in vivo. Am J Pathol 2002;160:501–509
30. Funatsu H, Yamashita H, Nakanishi Y, Hori S. Angiotensin II and vascular endothelial growth factor in the vitreous fluid of patients with proliferative diabetic retinopathy. Br J Ophthalmol 2002;86:311–315
31. Funatsu H, Yamashita H, Ikeda T, Nakanishi Y, Kitano S, Hori S. Angiotensin II and vascular endothelial growth factor in the vitreous fluid of patients with diabetic macular edema and other retinal disorders. Am J Ophthalmol 2002;133:537–543
32. Sealey JE, White RP, Laragh JH, Rubin AL. Plasma prorenin and renin in anephric patients. Circ Res 1977;41:17–21
33. Weinberger MH, Wade MB, Aoi W, Usa T, Dentino M, Luft F, Grim CE. An extrarenal source of "renin-like" activity in anephric man. Circ Res 1977;40(Suppl. 1):I1–I4
34. Franken AA, Derkx FH, Blankestijn PJ, Janssen JA, Mannesse CK, Hop W, Boomsma F, Weber R, Peperkamp E, De Jong PT, Schalekamp MA. Plasma prorenin as an early marker of microvascular disease in patients with diabetes mellitus. Diabete Metab 1992;18:137–143
35. Deinum J, Ronn B, Mathiesen E, Derkx FH, Hop WC, Schalekamp MA. Increase in serum prorenin precedes onset of microalbuminuria in patients with insulin-dependent diabetes mellitus. Diabetologia 1999;42:1006–1010
36. Danser AH, van den Dorpel MA, Deinum J, Derkx FH, Franken AA, Peperkamp E, de Jong PT, Schalekamp MA. Renin, prorenin, and immunoreactive renin in vitreous fluid from eyes with and without diabetic retinopathy. J Clin Endocrinol Metab 1989;68:160–167
37. Satofuka S, Ichihara A, Nagai N, Noda K, Ozawa Y, Fukamizu A, Tsubota K, Itoh H, Oike Y, Ishida S. (Pro)renin receptor promotes choroidal neovascularization by activating its signal transduction and tissue renin-angiotensin system. Am J Pathol 2008;173:1911–1918
38. Joussen AM, Poulaki V, Le ML, Koizumi K, Esser C, Janicki H, Schraermeyer U, Kociok N, Fauser S, Kirchhof B, Kern TS, Adamis AP. A central role for inflammation in the pathogenesis of diabetic retinopathy. Faseb J 2004;18:1450–1452
39. Ishida S, Usui T, Yamashiro K, Kaji Y, Amano S, Ogura Y, Hida T, Oguchi Y, Ambati J, Miller JW, Gragoudas ES, Ng YS, D'Amore PA, Shima DT, Adamis AP. VEGF164-mediated inflammation is required for pathological, but not physiological, ischemia-induced retinal neovascularization. J Exp Med 2003;198:483–489
40. Miyamoto K, Hiroshiba N, Tsujikawa A, Ogura Y. In vivo demonstration of increased leukocyte entrapment in retinal microcirculation of diabetic rats. Invest Ophthalmol Vis Sci 1998;39:2190–2194
41. Ruiz-Ortega M, Esteban V, Ruperez M, Sanchez-Lopez E, Rodriguez-Vita J, Carvajal G, Egido J. Renal and vascular hypertension-induced inflammation: role of angiotensin II. Curr Opin Nephrol Hypertens 2006;15:159–166
42. Otani A, Takagi H, Suzuma K, Honda Y. Angiotensin II potentiates vascular endothelial growth factor-induced angiogenic activity in retinal microcapillary endothelial cells. Circ Res 1998;82:619–628
43. Sakoda M, Ichihara A, Kaneshiro Y, Takemitsu T, Nakazato Y, Nabi AH, Nakagawa T, Suzuki F, Inagami T, Itoh H. (Pro)renin receptor-mediated activation of mitogen-activated protein kinases in human vascular smooth muscle cells. Hypertens Res 2007;30:1139–1146
44. Muller DN, Klanke B, Feldt S, Cordasic N, Hartner A, Schmieder RE, Luft FC, Hilgers KF. (Pro)renin receptor peptide inhibitor "handle-region" peptide does not affect hypertensive nephrosclerosis in Goldblatt rats. Hypertension 2008;51:676–681
45. Feldt S, Maschke U, Dechend R, Luft FC, Muller DN. The putative (pro)renin receptor blocker HRP fails to prevent (pro)renin signaling. J Am Soc Nephrol 2008;19:743–748
46. Feldt S, Batenburg WW, Mazak I, Maschke U, Wellner M, Kvakan H, Dechend R, Fiebeler A, Burckle C, Contrepas A, Jan Danser AH, Bader M, Nguyen G, Luft FC, Muller DN. Prorenin and renin-induced extracellular signal-regulated kinase 1/2 activation in monocytes is not blocked by aliskiren or the handle-region peptide. Hypertension 2008;51:682–688
47. Batenburg WW, Krop M, Garrelds IM, de Vries R, de Bruin RJ, Burckle CA, Muller DN, Bader M, Nguyen G, Danser AH. Prorenin is the endogenous agonist of the (pro)renin receptor. Binding kinetics of renin and prorenin in rat vascular smooth muscle cells overexpressing the human (pro)renin receptor. J Hypertens 2007;25:2441–2453
48. Imamura A, Mackenzie HS, Lacy ER, Hutchison FN, Fitzgibbon WR, Ploth DW. Effects of chronic treatment with angiotensin converting enzyme inhibitor or an angiotensin receptor antagonist in two-kidney, one-clip hypertensive rats. Kidney Int 1995;47:1394–1402
49. Janka HU, Warram JH, Rand LI, Krolewski AS. Risk factors for progression of background retinopathy in long-standing IDDM. Diabetes 1989;38:460–464
50. Teuscher A, Schnell H, Wilson PW. Incidence of diabetic retinopathy and relationship to baseline plasma glucose and blood pressure. Diabetes Care 1988;11:246–251