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

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**Objective.** Receptor-associated prorenin system (RAPS) refers to the pathogenic mechanisms whereby prorenin binding to its receptor dually activates 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 RAPS with diabetes-induced retinal inflammation.

**Research design and methods.** Long-Evans rats, C57BL/6 mice or 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 (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 ERK activation was analyzed by western blotting.

**Results.** Induction of diabetes led to significant increase in retinal expression of prorenin, but not (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 (pro)renin receptor in the diabetic retina, we utilized AT1-R-deficient mice in which 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 significant contribution of RAPS to the pathogenesis of diabetes-induced retinal inflammation, suggesting the possibility of (pro)renin receptor as a novel, molecular target for the treatment of diabetic retinopathy.
Since 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 angiotensin-converting enzyme (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 was shown to prevent the progression of diabetic retinopathy in UK Prospective Diabetes Study (UKPDS) (1); however, diabetic patients are generally characterized by low renin and high prorenin levels in the plasma, indicating that 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 tissue RAS in the diabetic retina is activated independently of circulatory RAS, although the precise mechanism for activating tissue RAS remains unclear.

(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. Since 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 tissue, but not circulatory, RAS. In addition, prorenin binding to its receptor is shown to cause RAS-independent signal transduction via phosphorylation of ERK (extracellular signal-regulated kinase) 1/2 in cells bearing (pro)renin receptor (4-7). Thus, we proposed the nomenclature “receptor-associated prorenin system (RAPS)” for the dual activation of 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 circulatory RAS, indicating a critical contribution of RAPS to the pathogenesis of diabetic nephropathy (6; 8-10).

Recently, we reported that AT1-R was expressed in the fibrovascular tissues surgically excised from human eyes with proliferative diabetic retinopathy (11) and 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 tissue RAS with diabetic retinopathy. This is supported by several recent reports showing that RAS contributes to various pathologic vascular conditions including inflammation and neovascularization via AT1-R signaling (18-21). However, the role of (pro)renin
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receptor as a trigger to activate tissue RAS in diabetes-induced retinal inflammation has not been defined. Although we have further shown that tissue RAS for promoting retinal inflammation (22) and neovascularization (23) is activated by nonproteolytically processed prorenin, whether (pro)renin receptor-mediated intracellular signaling, the other pathway of RAPS, is pathogenic in the eye has not been determined. In the present paper, we report the first evidence of significant relationship between 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 Co., Ltd., Osaka, Japan) at the age of 6 to 8 weeks were utilized. 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 ARVO (Association for Research in Vision and Ophthalmology) Statement for the Use of Animals in Ophthalmic and Vision Research. Animals received an intraperitoneal injection of streptozotocin (STZ; Sigma, St. Louis, MO) at the dose of 80 mg/kg body weight (BW) once for rats and three consecutive days for mice. Animals with plasma glucose levels greater than 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 (pro)renin receptor (25), we designed decoy peptides, NH₂-RILLKKMPSV-COOH(6; 9; 10; 22; 26) and NH₂-IPLKKMPS-COOH(8; 23) as rat and mouse (pro)renin receptor blockers (PRRB), respectively, and purified them by high-pressure liquid chromatography (HPLC) on a C-18 reverse-phase column, as described previously. 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 (pro)renin receptor and subsequent ERK activation was confirmed in our present (Supplemental Figs. 1 and 2, in an online appendix available at http://diabetes.diabetesjournals.org) and recent (27; 28) in vitro data. The specific inhibitory action of PRRB against 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 100ºC for 10 min and used as control peptide (CP). We also generated a scramble peptide (SP) NH₂-KPMLISKP-COOH for mouse PRRB as another negative control.

Treatment with PRRB, CP, SP or the AT1-R blocker losartan. Diabetic animals were intraperitoneally injected with vehicle (0.25% DMSO in phosphate-buffered saline [PBS]), CP (1.0 mg/kg), SP (1.0 mg/kg), PRRB (0.1 or 1.0 mg/kg), or the AT1-R blocker losartan (20 mg/kg; Cayman Chemical, Ann Arbor, MI) daily for 7 consecutive weeks immediately after the
establishment of diabetes (7 days after STZ injection) till the end of the study. Daily injection was based on our preliminary experiments showing the time course of serum concentration of PRRB injected to normal mice measured by BIAcore 2000 (GE Healthcare Bio-sciences KK, Tokyo, Japan; Supplemental Fig. 3, available in an online appendix). In our preliminary experiments, 20 mg/kg of losatan 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 (Con A) (Vector, Burlingame, CA), as described previously (29). After deep anesthesia, the chest cavity was opened and 20 or 27 G cannula (for rats or mice, respectively) was introduced into the left ventricle. After injection of PBS to remove erythrocytes and non-adherent leukocytes, 20 or 2 ml (for rats and mice, respectively) of FITC-conjugated Con A was perfused. After the eyes were enucleated, the retinas were flatmounted. The flatmounts were imaged using an epifluorescence microscope (IX71; Olympus, Tokyo, Japan) and the total number of Con A-stained adherent leukocytes per retina was determined.

Quantitative reverse transcription-polymerase chain reaction analyses for prorenin and (pro)renin receptor. We isolated total RNA from the retina with an RNeasy Mini Kit (Qiagen, Tokyo, Japan) and performed a real-time quantitative reverse transcription-polymerase chain reaction (RT–PCR) with the TaqMan One-Step RT–PCR Master Mix Reagents Kit; an ABI Prism 7700 HT Detection System (Applied Biosystems, Foster City, CA); and probes and primers for the rat genes that encode prorenin, (pro)renin receptor, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as described previously (9; 10; 26).

Immunohistochemistry for nonproteolytically activated prorenin and (pro)renin receptor. Rat eyes were fixed with 4% paraformaldehyde (PFA) 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 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 utilizing 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 (TSA) 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 ERK 1/2. The isolated retina was placed into the lysis buffer. After blocking nonspecific binding with 5% skim milk, polyvinylidene fluoride (PVDF) membranes were incubated with a mouse monoclonal antibody against phosphorylated ERK1/2 (1:1000; Cell Signaling Technology, Beverly, MA), total ERK1/2 (1:1000; Cell Signaling Technology) or α-tubulin (1:1000; Sigma, St. Louis, MO). Membranes were then incubated with biotin-conjugated secondary antibody (Jackson Immuno Research Laboratories Inc., 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 Health Care).

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 the 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 ($P < 0.01$) decrease in body weight and a significant ($P < 0.01$) increase in blood glucose, compared with age-matched non-diabetic controls (Table 1). Treatment with PRRB did not significantly ($P > 0.05$) change these metabolic parameters (Table 1).

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

Diabetes-induced activation of prorenin co-localized with (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 (pro)renin receptor. Immunoreactivity of activated prorenin in the retinal vessels was negligible in non-diabetic controls (Fig. 1C), substantially enhanced in diabetes (Fig. 1D), and abrogated by PRRB application to the background signal (Fig. 1E). In contrast, immunoreactivity of (pro)renin receptor (Fig. 1F) was unaltered by induction of diabetes (Fig. 1G) or treatment with PRRB (Fig. 1H).

PRRB treatment suppressed diabetes-induced retinal leukocyte adhesion. The retinal adherent leukocytes were imaged by perfusion labeling with FITC-coupled Con A. Leukocyte counts were evaluated in the whole retina from the
posterior retina around the optic disc (Fig. 2A-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. 2B) and CP-treated (Fig. 3C) diabetic rats than in normal age-matched controls (Fig. 3A). PRRB-treated diabetic rats (Fig. 2D) showed a significant decrease in the leukocyte count in a dose-dependent fashion, compared with vehicle-treated ($P < 0.05$) or CP-treated ($P < 0.01$) diabetic rats (Fig. 2E).

**PRRB treatment suppressed diabetes-induced upregulation of retinal VEGF and ICAM-1 production.** Retinal protein levels of VEGF (Fig. 3A) and ICAM-1 (Fig. 3B) 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. 3A 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, SP, the AT1-R blocker losartan, PRRB, and losartan plus PRRB. Compared with vehicle (Fig. 4A) or SP treatment, the inhibitory effect of PRRB (Fig. 4C, 71.3% inhibition) on diabetic retinal inflammation was more potent than that of losartan alone (Fig. 4B, 32.4% inhibition) and was equivalent to that of losartan plus PRRB (Fig. 4D), showing that PRRB in combination with losartan caused little or no additive effect compared with PRRB alone (Fig. 4E). Thus, PRRB was suggested to inhibit both angiotensin II-dependent (32.4% contribution) and -independent actions ($71.3 - 32.4 = 38.9$% contribution).

**RAS-independent (pro)renin receptor signaling contributed to diabetes-induced retinal leukocyte adhesion.** In order to clarify the role of RAS-independent intracellular signaling via (pro)renin receptor, we used mice in which 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 non-diabetic 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 to non-diabetic AT1-R-deficient mice (Fig. 5A), induction of diabetes (Fig. 5B) 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 mice (Fig. 5C) at the dose of 1.0 mg/kg than in vehicle-treated diabetic AT1-R-deficient mice (Fig. 5D). These two data with pharmacological blockade (Fig. 4) and genetic ablation (Fig. 5) of AT1-R indicated dual contribution of tissue RAS (32.4% and 27.3%, respectively) and RAS-independent (pro)renin receptor signaling (38.9% and 46.6%, respectively) 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 (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. 6A). Relative phosphorylation of ERK1/2, increased by inducing diabetes, was significantly ($P < 0.01$) suppressed by PRRB application, while no significant ($P > 0.05$) difference was detected in total ERK1/2 protein levels (Fig. 6B).

RAS-independent (pro)renin receptor signaling contributed to retinal expression of VEGF but not ICAM-1. To examine whether RAS-independent intracellular signaling via (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 to non-diabetic AT1-R-deficient mice, induction of diabetes significantly enhanced retinal expression of VEGF ($P < 0.01$) (Fig. 7A) and ICAM-1 ($P < 0.05$) (Fig. 7B). PRRB application to AT1-R-deficient diabetic mice led to significant suppression of VEGF ($P < 0.05$) (Fig. 7A), but not ICAM-1 ($P > 0.05$) (Fig. 7B), compared with vehicle treatment to AT1-R-deficient diabetic mice.

**DISCUSSION**

The present study reveals, for the first time to our knowledge, several important findings concerning the role of (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 co-localized 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 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 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 tissue RAS with diabetic retinopathy, the precise mechanism for activating tissue RAS in the diabetic retina has not been determined. In parallel, little has been known about the bioactivity of prorenin molecule per se other than functioning as the inactive precursor of renin. Bilateral nephrectomy resulted in significant decline of plasma renin, but not prorenin, to the undetectable level, suggesting that the
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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) has indicated the possibility of prorenin as 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 non-proliferative 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. 1A), whereas (pro)renin receptor levels were unaltered (Fig. 1B). 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. 1C-H), suggest the significance of the ligand-based regulation in the activation of RAPS. Importantly, nonproteolytically activated prorenin was localized in diabetic retinal vessels bearing (pro)renin receptor (Fig. 1D and G), showing that the initiating step of tissue RAS is prepared in the diabetic retina.

Molecular and cellular mechanisms underlying the pathogenesis of diabetic retinopathy are not fully understood; however, increasing evidence suggested the involvement of inflammatory processes including cytokine upregulation and leukocyte infiltration, causing diabetic retinopathy being 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 enhancement of leukocyte-endothelial 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 pro-inflammatory 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 is 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 recently showed that tissue RAS enhanced retinal expression of these inflammatory molecules and subsequent leukocyte adhesion to the
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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 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 RAPS, (pro)renin receptor-mediated signal transduction and tissue RAS activation, in the pathogenesis of diabetic retinopathy. We recently showed 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 as compared to wild-type diabetes, indicating a significant role of tissue RAS in diabetic nephropathy. Surprisingly, these renal events in AT1-R-deficient diabetes 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 RAPS were involved in the pathogenesis of diabetic nephropathy (8). In the present study as well, we administered PRRB to AT1-R-deficient diabetic mice and confirmed the significant role of intracellular signaling via (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 (pro)renin receptor (Fig. 6). Out 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 MCP-1 out of several inflammatory and angiogenic molecules mediated by AT1-R (37). These new findings (Fig. 4-7) clarified molecular and cellular mechanisms mediated by RAS-independent intracellular signaling via (pro)renin receptor in diabetes-induced retinal inflammation. In addition to our recent reports showing that 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 RAPS with inflammation in the eye (Fig. 8).

In the present data, there was a discrepancy between in vivo (Figs. 1-7 and Suppl Fig. 3) and in vitro (Suppl Figs. 1-2) doses of PRRB. In vivo, intraperitoneal injection of PRRB at the dose of 1.0 mg/kg/day led to serum concentration of 110
ng/ml (approximately 100 nM) or lower, which was cleared within 24 hours (Suppl Fig. 3). Since the maximal concentration of PRRB in the serum is 50-100 times higher than the physiological level of endogenous prorenin (1-2 nM), the currently used dose of 1.0 mg/kg/day is thought to be sufficient enough to inhibit the in vivo binding of prorenin to the receptor. Indeed, a lower dose of 0.1 mg/kg/day (presumably 10 nM 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 µM) were required to suppress prorenin-stimulated ERK activation (Suppl Fig. 2). This would be attributable at least in part to the in vitro property of cellular localization of (pro)renin receptor. In cultured vascular smooth muscle cells (43), indeed, (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 tissue RAS does not originally play any role or in vitro (46; 47) models applied with too low concentration (1 µM or lower) of PRRB. The glomerulosclerosis in the clipped kidney in the rat model of 2-kidney, 1-clip (2K1C) hypertension was already 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 circulatory but not tissue RAS), causing hypertension-induced cardiac and renal damages (45). Reasonably, PRRB did not alter these pathologic conditions due to 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, since (pro)renin receptor is present in the major organs but not in the circulation, PRRB does not affect circulatory RAS or systemic blood pressure (9; 10). Interestingly, PRRB treatment 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 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.

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### Table 1

#### Systemic Data

|          | Rats |               |               |               |               |
|----------|------|---------------|---------------|---------------|---------------|
|          | Normal | DM |               |               |               |
|          | Vehicle | CP | PRRB |               |               |
| Number   | 7      | 12 | 6 | 7 |               |
| Body weight (g) | 448.6±13.5 | 280.2±9.3* | 271.4±15.7* | 289.2±24.1* |               |
| Blood glucose (mg/dl) | 139±5 | 534±22* | 537±39* | 531±33* |               |

#### Mice

|          | Wild type | AT1-R KO |               |               |               |               |               |               |               |               |               |
|----------|------------|----------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|
|          | Normal | DM | non DM | DM |               |               |               |               |               |               |               |
|          | Vehicle | SP | Losartan | PRRB | Losartan+PRRB | Vehicle | PRRB |               |               |               |               |
| Number   | 12 | 17 | 6 | 5 | 17 | 4 | 10 | 10 | 11 |               |               |               |
| Body weight (g) | 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) | 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 non DM.
FIGURE LEGENDS

Fig. 1. Upregulation of prorenin expression and tissue localization of prorenin and (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 (n=8). (B) (Pro)renin receptor mRNA levels were unchanged following induction of diabetes (n=8). Immunostaining for nonproteolytically activated prorenin (C-E, green fluorescence) and (pro)renin receptor (F-H, red fluorescence) in retinal vessels. **P < 0.01.

Fig. 2. Inhibitory effect of PRRB on retinal leukocyte adhesion. Flatmounted retinas from normal (A) and diabetic rats treated with vehicle (B), CP (C) and 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 CP-treated diabetic rats. (n=5-13). **P < 0.01, *P < 0.05.

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 (n=8-11). **P < 0.01, *P < 0.05.

Fig. 4. PRRB was more potent in inhibiting diabetes-induced retinal leukocyte adhesion than the AT1-R blocker losartan. Flatmounted retinas from diabetic mice treated with vehicle (A), losartan (B), PRRB (C) and losartan plus PRRB (D). Scale bars, 50 µm. (E) Compared to 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.

Fig. 5. RAS-independent (pro)renin receptor-mediated intracellular signaling contributes to diabetes-induced retinal leukocyte adhesion. Flatmounted retinas from non-diabetic (A) and diabetic AT1-R-deficient mice treated with vehicle (B) and PRRB (C). Scale bars, 100 µm. (D) The number of retinal adherent leukocytes. Compared with diabetic wild-type animals, diabetic AT1-R-deficient mice exhibited a significant reduction of the number of adherent leukocytes. PRRB treatment to diabetic AT1-R-deficient mice showed significantly fewer adherent leukocytes than did vehicle administration (n=6-8). **P < 0.01.

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

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

Fig. 8. A scheme showing the significant involvement of RAPS with the pathogenesis of diabetes-induced retinal inflammation.
(Pro)renin receptor-mediated retinal inflammation in diabetes

Figure 1.

A

B

C

D

E

F

G

H

Normal

Vehicle

PRRB

Normal

Vehicle

PRRB

DM
Figure 2.
Figure 3.

(A) VEGF

(B) ICAM-1
Figure 4.
Figure 5.

(A) AT1-R KO

non DM

DM+Vehicle

DM+PRRB

(D) Adherent leukocytes

| Condition | non DM | DM | Vehicle | PRRB | non DM | DM | Vehicle | PRRB |
|-----------|--------|----|---------|------|--------|----|---------|------|
| WT        |        |    |         |      |        |    |         |      |
| AT1-R KO  |        |    |         |      |        |    |         |      |

** ** ** **
Figure 6.

A

B

Relative ERK 1/2 phosphorylation

***  ***
Figure 7.

A

VEGF

**

* (*%)

Vehicle PRRB

DM non DM

AT1-R KO

B

ICAM-1

*

N.S. (*%)

Vehicle PRRB

DM non DM

AT1-R KO
Figure 8.