

Receptor-associated prorenin system in the pathogenesis of retinal diseases

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1. ABSTRACT

Receptor-associated prorenin system (RAPS) refers to the pathogenic mechanisms whereby prorenin binding to (pro)renin receptor [(P)RR] dually activates tissue renin-angiotensin system (RAS) and RAS-independent intracellular signaling through the receptor. Although we found significant involvement of angiotensin II type 1 receptor (AT1-R) in intraocular inflammation and neovascularization, central pathologies of age-related macular degeneration and diabetic retinopathy, the association of RAPS with these vision-threatening disorders has not been defined. (P)RR blockade to murine disease models led to significant suppression of laser-induced choroidal neovascularization and diabetes-induced retinal inflammation together with the upregulation of intercellular adhesion molecule (ICAM)-1, monocyte chemotactic protein (MCP)-1 and vascular endothelial growth factor (VEGF). Either the genetic ablation or the pharmacological blockade of AT1-R exhibited significant reduction of choroidal and retinal abnormalities, both of which were further suppressed by (P)RR blockade. (P)RR blockade inhibited ERK activation and the production of VEGF and MCP-1, but not ICAM-1, in AT1-R-deficient mice with retinal and choroidal disorders. These recent findings indicate significant contribution of RAPS to the pathogenesis of age-related macular degeneration and diabetic retinopathy.

2. INTRODUCTION

(Pro)renin receptor [(P)RR], also called ATP6AP2, was identified as a single transmembrane protein consisting of 350 amino acids. The fragment (P)RR has been shown to be associated with vacuolar-type H⁺-ATPase (V-ATPase) and required for Wnt/ β -catenin signaling pathway (1). In addition to its role for vesicular acidification, (P)RR interacts with its ligand 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 (Figure 1A). Since the membrane-bound (P)RR is reported to exist in the major organs but not in the circulation (2), the nonproteolytic activation of prorenin is hypothesized to play a critical role in the activation of tissue, but not circulatory, renin-angiotensin system (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 (P)RR (2-5). Thus, we proposed the nomenclature “receptor-associated prorenin system (RAPS)” for the dual activation of tissue RAS and RAS-independent signaling pathway. In streptozotocin (STZ)-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

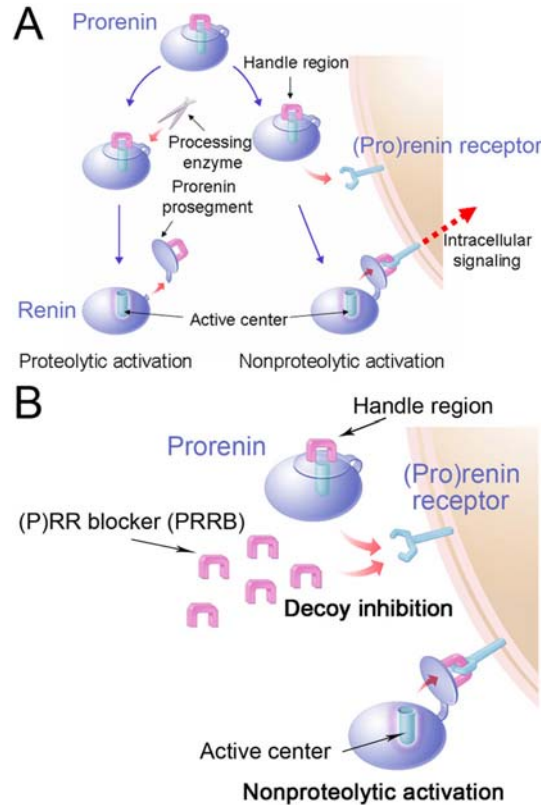


Figure 1. Two major pathways of prorenin activation and PRRB as a decoy for prorenin. (A) Proteolytic activation of prorenin by processing enzymes versus nonproteolytic activation by (pro)renin receptor binding to the handle region of the prorenin prosegment. (B) Nonproteolytic activation of prorenin is blocked by a decoy peptide, PRRB, which competitively binds to (pro)renin receptor. Reproduced with permission from (11, 12).

critical contribution of RAPS to the pathogenesis of diabetic nephropathy (4, 6-8).

Choroidal neovascularization (CNV) and diabetes-induced retinal inflammation is a hallmark of vision-threatening disorders, including age-related macular degeneration (AMD) and diabetic macular edema, both of which are the major causes of central vision loss. We reported that tissue RAS was upregulated and angiotensin II type 1 receptor (AT1-R) signaling plays crucial roles in choroidal and retinal neovascularization (9, 10) by inducing several angiogenic and inflammatory factors. Although we further showed that tissue RAS for promoting retinal inflammation (11) and neovascularization (12) is triggered by (P)RR-induced nonproteolytic activation of prorenin, it was not determined whether (P)RR-mediated intracellular signaling, the other pathway of RAPS, is pathogenic in the eye.

We therefore hypothesized that prorenin binding to its receptor promotes CNV and diabetes-induced retinal inflammation by dually activating tissue RAS and RAS-independent ERK pathway as an intracellular signaling via the receptor. Recently, we reported the first evidence of significant relationship between RAPS and these choroidal and retinal disorders together with underlying molecular and cellular mechanisms (13, 14).

3. BLOCKADE OF PRORENIN BINDING TO (P)RR

To cover the handle region (positions 11-15) of the prorenin molecule, which is the binding site of (P)RR (15), we designed decoy peptides, $\text{NH}_2\text{-RILLKKMPSV-COOH}$ and $\text{NH}_2\text{-IPLKKMPS-COOH}$ as rat and mouse (P)RR blockers (PRRBs) (Figure 1B), respectively, and purified them by high-pressure liquid chromatography on a C-18 reverse-phase column, as described previously (4, 6-8, 11, 16). The specific inhibitory action of PRRB against prorenin binding with (P)RR and subsequent ERK activation was confirmed in recent *in vitro* data (14, 17, 18). The specific inhibitory action of PRRB against tissue RAS or RAPS *in vivo* was also confirmed in other reports (4, 6-8, 11-14, 16). As a negative control, rat PRRB was inactivated by heat denaturation at 100°C for 10 min and used as control peptide (CP). We also generated a scramble peptide (SP) $\text{NH}_2\text{-KPMLISKP-COOH}$ for mouse PRRB as another negative control.

4. PATHOLOGIC ROLE OF (P)RR IN THE EYE

4.1. AMD

4.1.1. CNV and tissue RAS

AMD, the most common cause of blindness in developed countries, is complicated by CNV leading to severe vision loss due to hemorrhage and exudation from

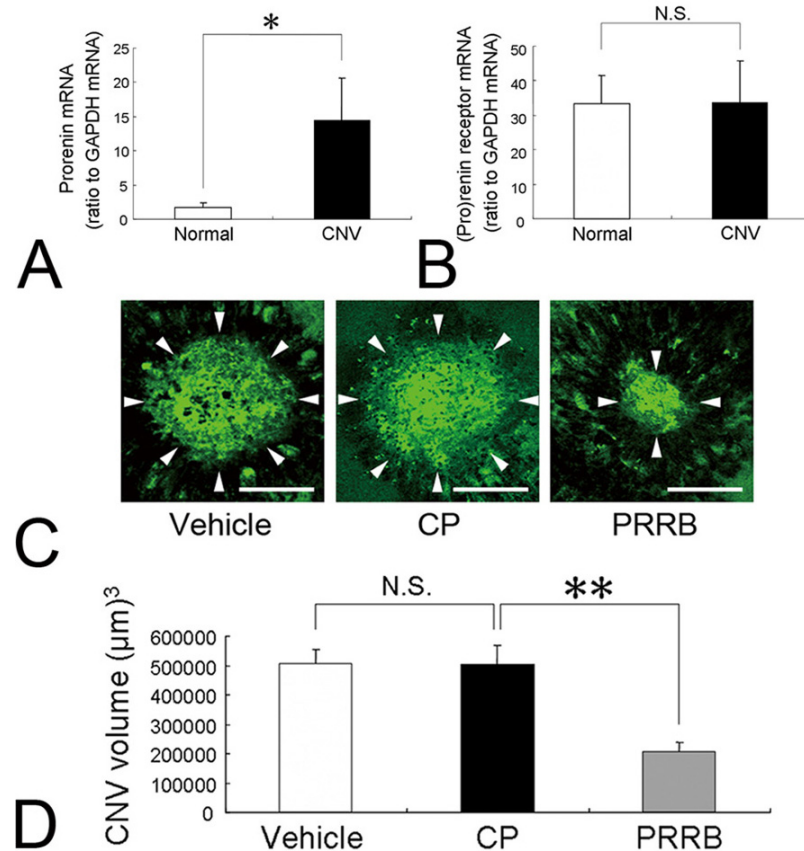


Figure 2. Prorenin expression is upregulated in CNV and PRRB inhibits CNV. (A) Upregulation of prorenin mRNA levels in the RPE-choroid complex by inducing CNV (n=6-7). (B) (P)RR mRNA levels were unchanged following CNV induction (n=6-7). (C) Flatmounted choroids from vehicle-, CP-, and PRRB-treated mice. (D) The graph showing the CNV volume. PRRB application led to significant suppression of CNV, compared with vehicle or CP treatment. Arrowheads in (C) indicate lectin-stained CNV tissues (n=33-37). *P < 0.05, **P < 0.01. Scale bars = 100 μm . Reproduced with permission from (13).

the immature new vessels (19, 20). Epidemiologic risk factors for AMD were reported to include hypertension, dyslipidemia, and atherosclerosis, all of which are related to the metabolic syndrome (21, 22). AT1-R signaling was shown to play a significant role in various pathologic processes such as angiogenesis and inflammation, both of which complicate the metabolic syndrome (23-26). CNV proved to be an inflammatory disorder depending on intercellular adhesion molecule (ICAM)-1, monocyte chemoattractant protein (MCP)-1 and vascular endothelial growth factor (VEGF) (27). We showed that AT1-R-mediated upregulation of these inflammatory and angiogenic molecules is required for the development of CNV (9).

4.1.2. Pathologic roles of (P)RR in CNV

We revealed several important findings concerning the role of (P)RR in CNV generation (13). First, CNV development was associated with upregulation of prorenin mRNA expression, but not (P)RR, in the retinal pigment epithelium (RPE)-choroid complex and PRRB treatment showed a significant decrease in the CNV volume, indicating that prorenin binding with its receptor contributes to CNV (Figure 2). Second, the cellular and

molecular mechanisms in the PRRB-induced suppression of CNV included the inhibitory effects on macrophage infiltration into CNV, angiotensin II generation and the upregulated expression of inflammatory and angiogenic molecules such as ICAM-1, MCP-1, VEGF, VEGF receptor (VEGFR)-1 and VEGFR-2, all of which were downstream molecules of angiotensin II (Figure 3) (9). Although the detailed molecular and cellular mechanisms underlying CNV are not fully clarified, ICAM-1 expression (28, 29) and macrophage infiltration (9, 27) were observed in CNV tissues from human eyes with AMD and the laser-induced murine model, suggesting the close association of inflammation with the progression of CNV. Pharmacologic depletion of macrophages (30, 31) or genetic ablation of CCR2 (32), a receptor for MCP-1, was shown to result in the reduction of CNV, suggesting that macrophages, recruited by MCP-1 released from RPE or vascular endothelial cells, facilitate the development of CNV by producing VEGF. In concert with our previous data (9), PRRB-induced suppression of CNV indicates that tissue RAS is activated during CNV by (P)RR-mediated nonproteolytic activation of prorenin, leading to AT1-R signaling-mediated upregulation of CNV-related inflammatory molecules.

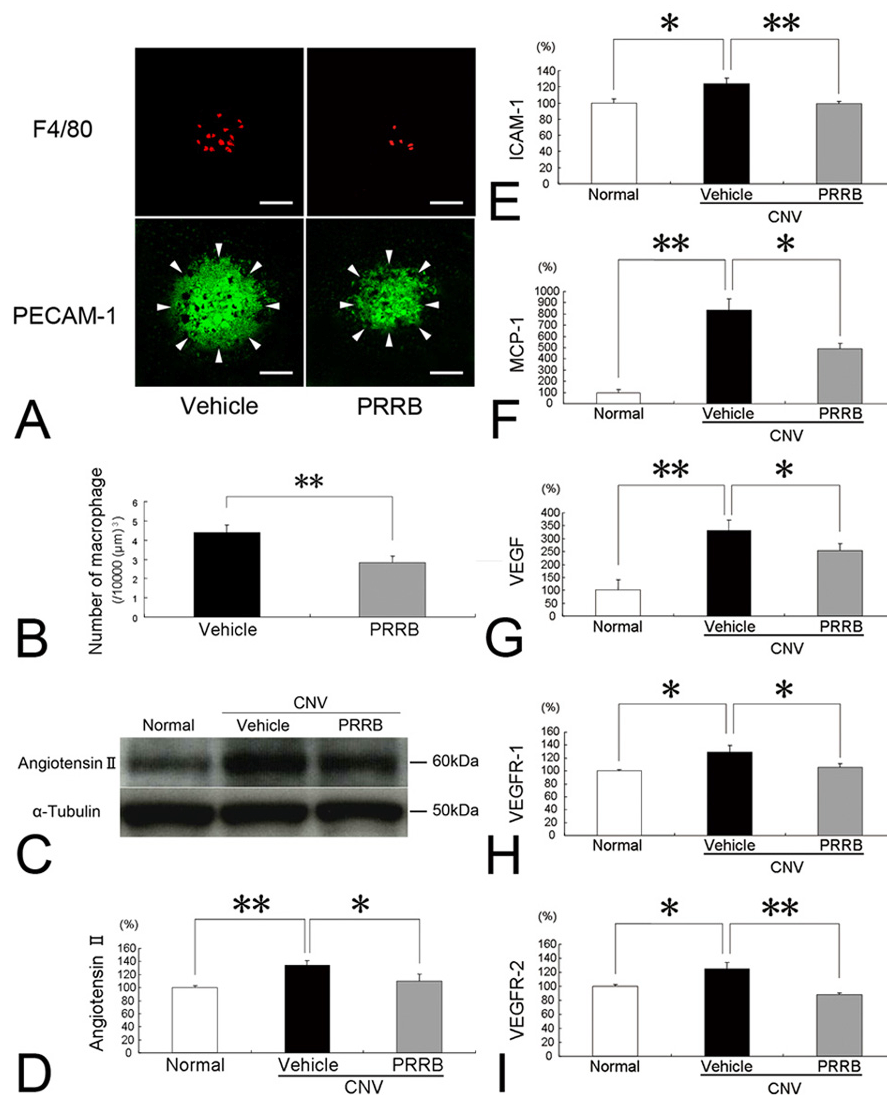


Figure 3. PRRB inhibits macrophage infiltration and RPE-choroidal production of angiotensin II and CNV-related inflammatory molecules. F4/80-positive macrophages (A, top) and PECAM-1-stained CNV (A, bottom) were evaluated and the volume-adjusted number of macrophages is shown in the graph (B). PRRB led to significant suppression of macrophage infiltration into CNV ($n = 20$). Scale bars = 50 μm . C-D: RPE-choroidal generation of angiotensin II was significantly reduced by treatment with PRRB. PRRB significantly suppressed the protein levels of ICAM-1(E), MCP-1(F), VEGF(G), VEGFR-1(H), and VEGFR-2 (I) in the RPE-choroid ($n=4-9$). * $P < 0.05$, ** $P < 0.01$. Reproduced with permission from (13).

To elucidate the involvement of RAPS comprising both (P)RR-mediated signal transduction and tissue RAS activation, we studied the role of RAS-independent (P)RR signaling in CNV generation (Figure 4). (P)RR signaling was shown to contribute to the pathogenesis of diabetic nephropathy using AT1-R-deficient mice (6). AT1-R-deficient mice with streptozotocin (STZ)-induced diabetes exhibited reduced proteinuria and glomerulosclerosis in the early phase as compared to STZ-treated wild-type mice, 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 diabetic wild-type mice. 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. We administered PRRB to CNV mice receiving the AT1-R blocker losartan or genetically deficient in AT1-R or angiotensinogen (AGT), and these three different methods for deactivating RAS confirmed the significant role of intracellular signaling via (P)RR in the development of CNV (Figure 4A, B). The data were compatible with the result of parallel experiments showing that macrophage infiltration into CNV was also suppressed by PRRB in AGT-deficient mice (Figure 4C, D). PRRB application to AT1-R-deficient mice

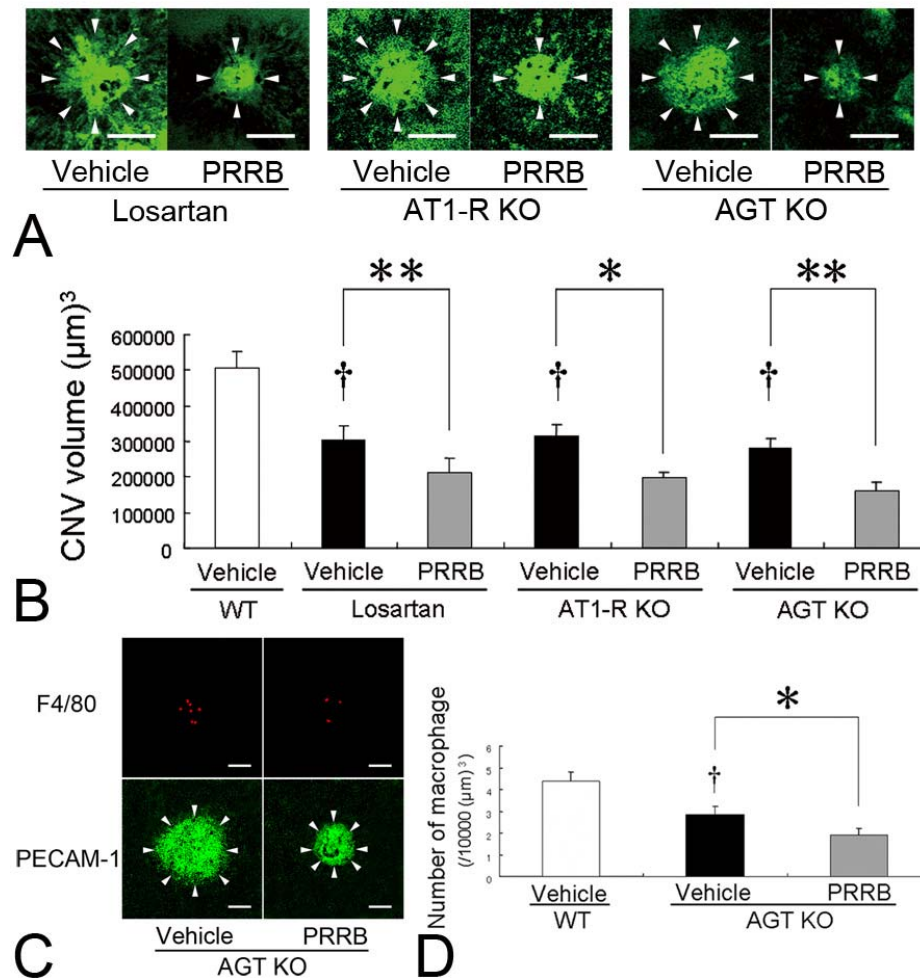


Figure 4. RAS-independent (P)RR-mediated intracellular signaling contributes to CNV development and macrophage infiltration. The graph showing the choroidal flatmounts (A) and the CNV volume (B). PRRB treatment further induced a significant decrease in the CNV volume in losartan-treated, AT1-R-deficient and AGT-deficient mice. Arrowheads in (A) indicate lectin-stained CNV tissues (n=23-40). Scale bars = 100 μm . F4/80-positive macrophages (C, top) and PECAM-1-stained CNV (C, bottom) were evaluated in AGT-deficient mice, and the volume-adjusted number of macrophages is shown in the graph (D). PRRB further caused significant suppression of macrophage infiltration (n=14-17). †P < 0.01 compared with vehicle wild-type, *P < 0.05, **P < 0.01. Scale bars = 50 μm . Reproduced with permission from (13).

with CNV led to significant suppression of ERK activation (Figure 5A, B). Out of the CNV-related molecules, the expression of which was inhibited by PRRB (Figure 3 E-I), MCP-1 (Figure 5C) and VEGF (Figure 5D), but not ICAM-1, VEGFR-1 or VEGFR-2 (data not shown), were also regulated by (P)RR signaling per se. These results revealed that molecular and cellular mechanisms mediated by RAS-independent intracellular signaling via (P)RR is involved in CNV generation (Figures 4, 5). This is the first report to show that RAPS is associated with inflammation and angiogenesis in the eye (13), in consistence with previous reports showing that RAPS contributes to glomerulosclerosis in the kidney and fibrosis in the heart (4, 6, 7, 16). Moreover, we reported that nonproteolytic activation of prorenin selectively accelerates pathologic, but not physiologic, retinal neovascularization via the

inflammatory processes in ischemia-induced retinal neovascularization (12). Recently, PRRB was also shown to exert anti-angiogenic and anti-inflammatory effects in ischemia-induced retinal neovascularization (33), which confirmed our previous data; however, PRRB-induced injury to retinal neurons was simultaneously observed in contrast with its protective effect on the retinal vasculature.

4.2. Diabetic retinopathy

4.2.1. Diabetic retinopathy and tissue RAS

AT1-R protein was expressed in the fibrovascular tissues surgically excised from human eyes with proliferative diabetic retinopathy and tissue RAS is activated in the retina of mice with STZ-induced diabetes (34). Diabetic retinopathy proved to be an inflammatory disorder depending on VEGF and ICAM-1 (35-39). We

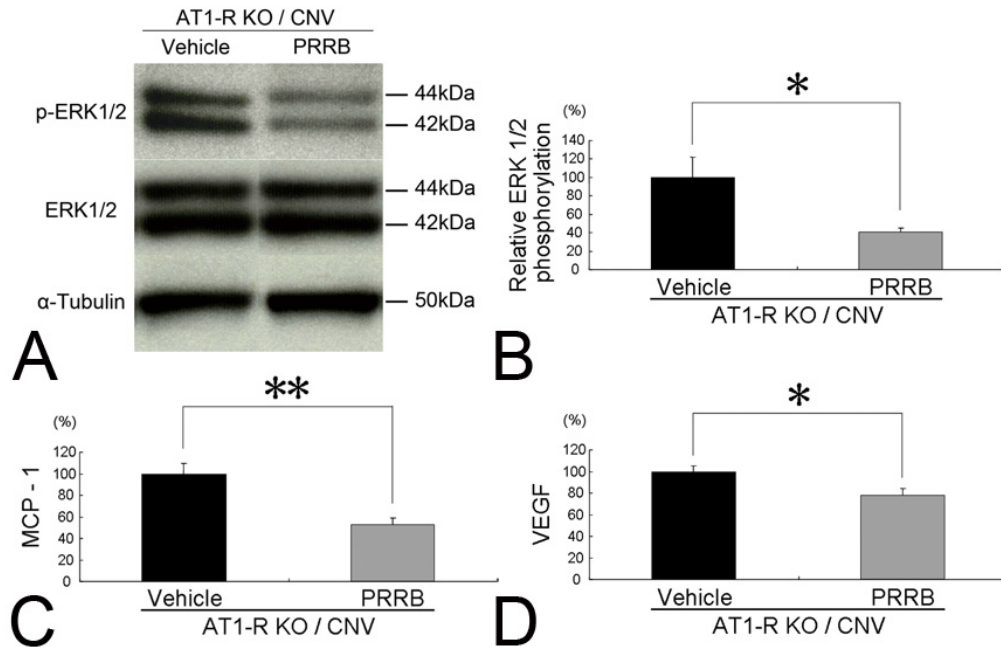


Figure 5. RAS-independent (P)RR-mediated intracellular signaling contributes to CNV-related activation of ERK1/2 and expression of inflammatory molecules. (A-B) Immunoblot analysis for phosphorylated and total levels of ERK1/2 in AT1-R-deficient mice with CNV. PRRB suppressed relative phosphorylation of ERK1/2. (C-D) Effects by blocking intracellular signaling via (P)RR on protein levels of CNV-related molecules. MCP-1 (C) and VEGF (D) levels were significantly suppressed with PRRB (n=9-11). *P < 0.05, **P < 0.01. Reproduced with permission from (13).

showed that diabetes-induced upregulation of these inflammatory and angiogenic molecules is mediated by AT1-R signaling and required for diabetes-induced retinal leukocyte adhesion (34), indicating the association of tissue RAS with diabetic retinopathy. This is supported by several studies showing that RAS contributes to various pathologic vascular conditions including inflammation and neovascularization via AT1-R signaling (40-43).

4.2.2. Pathologic roles of (P)RR in diabetes-induced retinal inflammation

Recent studies revealed that (P)RR was localized to vascular endothelial cells, pericytes, glia and ganglion cells in the rodent retina (14, 33) and human RPE cells (44). Photoreceptor morphology was abnormal in (P)RR conditional knockout mice (A.K., S.I. *et al.*; unpublished data), suggesting that (P)RR has a physiologic role during photoreceptor development. On the other hand, microvascular expression of (P)RR in the retina led us to hypothesize its involvement with the pathogenesis of diabetic retinopathy. We recently reported that several important findings concerning the role of (P)RR in diabetes-induced retinal inflammation (14). 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. The molecular mechanisms in the suppression of retinal leukocyte adhesion proved to include the inhibitory effects of PRRB on retinal expression of VEGF and ICAM-1, both of which are known as key

factors responsible for diabetes-induced retinal inflammation (Figure 6).

Molecular and cellular mechanisms underlying the pathogenesis of diabetic retinopathy are not fully understood; however, increasing evidence suggested that the involvement of inflammatory processes including cytokine upregulation and leukocyte infiltration, causing diabetic retinopathy being regarded as an inflammatory disease (36, 39, 45-47). Retinal vasculature in diabetes is accompanied by inflammatory cell adhesion (48), which triggers vascular hyperpermeability (36) and pathologic neovascularization (47). 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 and experimentally induced diabetes demonstrated that retinal ICAM-1 expression was elevated together with leukocyte adhesion and infiltration (36, 45). 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 (37), but also during the nonproliferative stage characterized by diabetic macular edema (38). Interestingly, angiotensin II levels were elevated and correlated with VEGF levels in the vitreous fluid of patients with diabetic macular edema (49). Angiotensin II was shown to induce ICAM-1 (50) and VEGF (51) through AT1-R in previous *in vivo* and *in vitro* studies. Also in the murine model of STZ-induced diabetes,

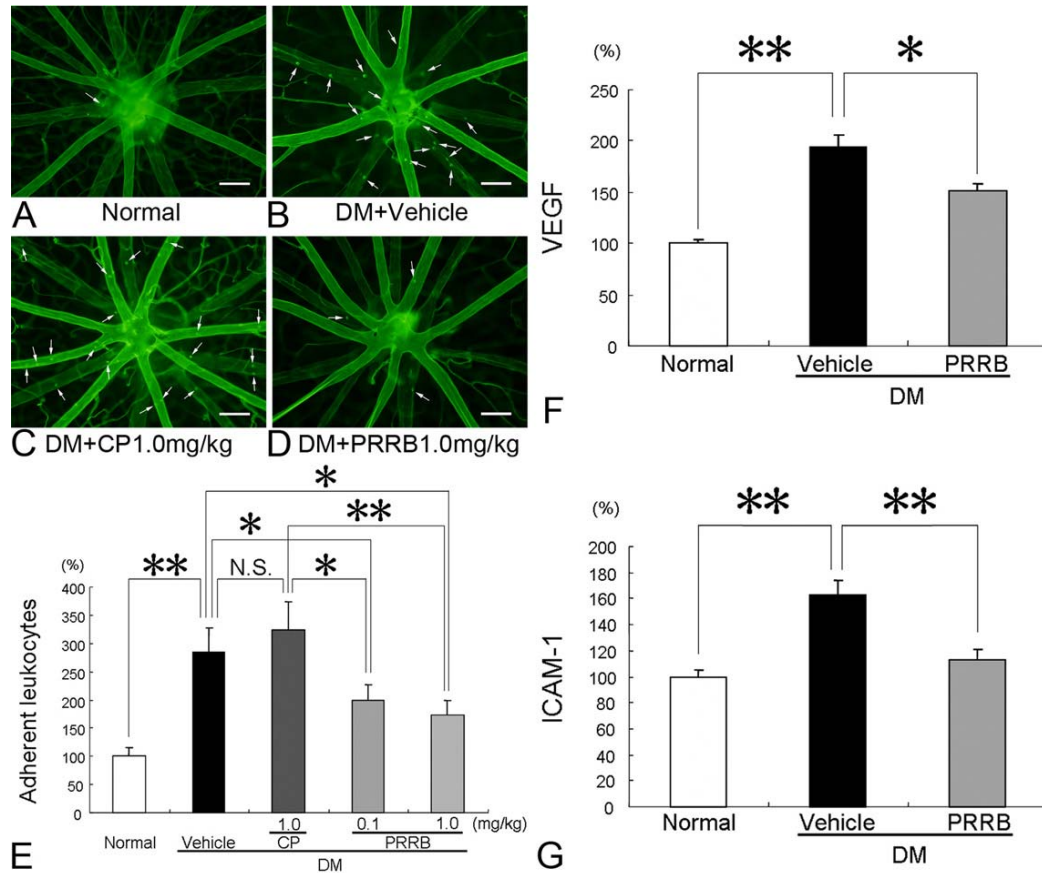


Figure 6. PRRB suppresses retinal leukocyte adhesion and retinal expression of inflammatory molecules. Flatmounted retinas from normal (A) and diabetic rats treated with vehicle (B), CP (C) and PRRB (D). Scale bars = 100 μ m. (E) The number of retinal adherent leukocytes. PRRB-treated diabetic rats showed significantly fewer adherent leukocytes than did vehicle- or CP-treated diabetic rats ($n=5-13$). PRRB significantly suppressed retinal protein levels of VEGF (F) and ICAM-1 (G) analyzed by ELISA, both of which were elevated after induction of diabetes ($n=8-11$). * $P < 0.05$, ** $P < 0.01$. Reproduced with permission from (14).

we showed that 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 (34). In concert with the previous data, the currently observed PRRB-induced suppression of diabetes-induced retinal inflammation (Figure 6) indicates that tissue RAS is activated in the diabetic retina by (P)RR-mediated nonproteolytic activation of prorenin, leading to AT1-R signaling-mediated VEGF and ICAM-1 upregulation and retinal leukocyte adhesion.

We further revealed the involvement of RAPS in the pathogenesis of diabetic retinopathy. The use of two different methods of inactivating AT1-R confirmed that PRRB functioned to inhibit both RAS-dependent and -independent mechanisms underlying retinal leukocyte adhesion (Figure 7). We administered PRRB to diabetic mice treated with losartan (Figure 7A) or AT1-R-deficient diabetic mice (Figure 7B) and confirmed the significant role of intracellular signaling via (P)RR in the diabetes-induced retinal leukocyte adhesion. PRRB application to

AT1-R-deficient diabetic mice led to significant suppression of ERK activation, a known key intracellular signaling through (P)RR (Figure 8A, B). Of diabetes-related inflammatory molecules, the expression of which was inhibited by PRRB (Figure 6F, G), VEGF, but not ICAM-1, was also regulated by (P)RR signaling per se (Figure 8C, D). The data are supported by and consistent with our recent report on CNV showing that (P)RR signaling selectively induces VEGF and MCP-1 out of several inflammatory and angiogenic molecules mediated by AT1-R (13). Furthermore, a recent study showed the involvement of (P)RR in the pathogenesis of diabetic nephropathy by the induction of inflammatory cytokines independently of renal RAS (52). Combined with these results, RAS-independent intracellular signaling pathway through (P)RR is associated with inflammation in diabetes-induced organ damage including retinopathy.

5. PERSPECTIVE

Although hypertension is a known risk factor for AMD and diabetic retinopathy (53, 54), there are indeed a

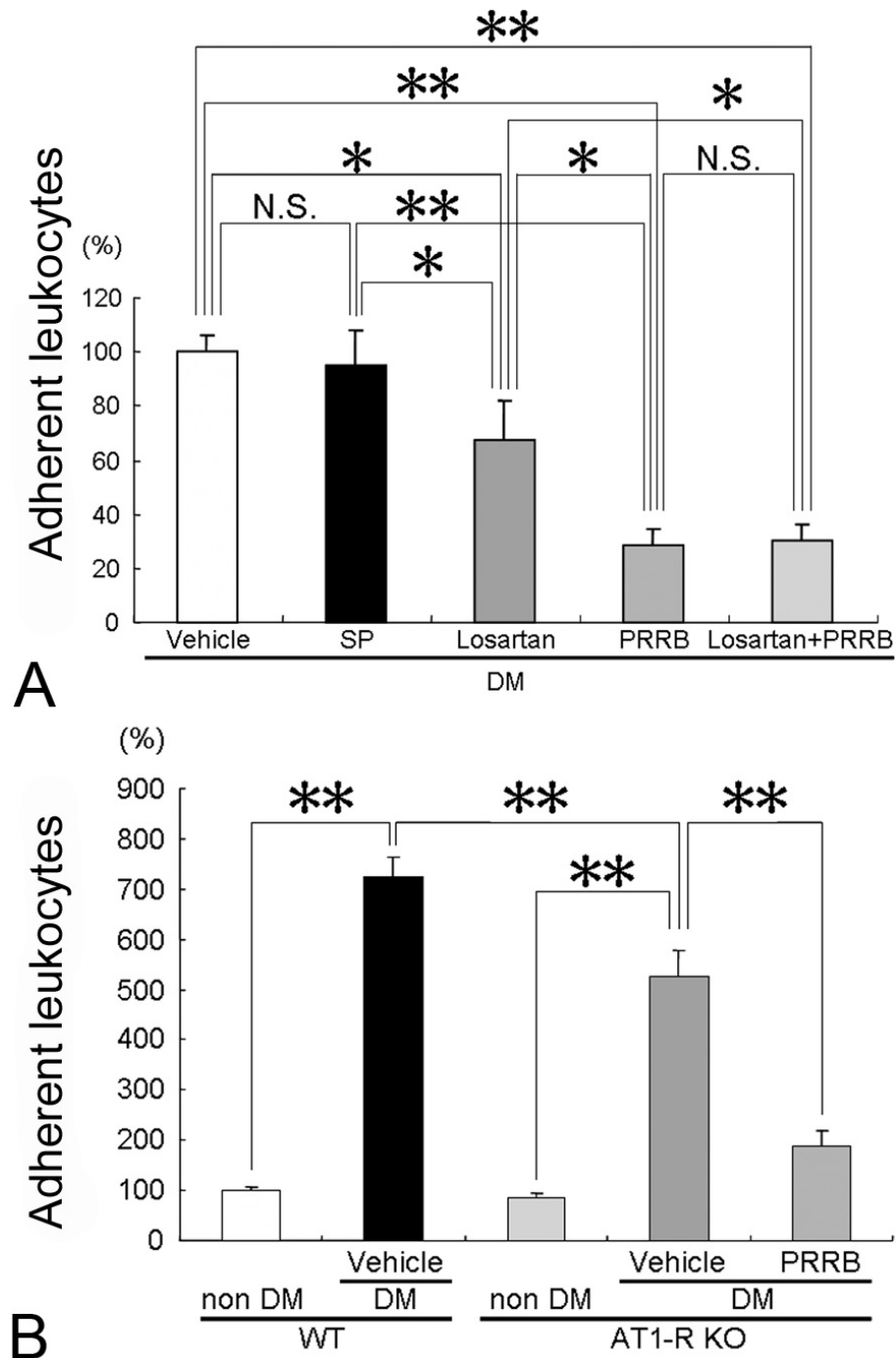


Figure 7. PRRB is more potent in inhibiting diabetes-induced retinal leukocyte adhesion than the AT1-R blocker losartan and RAS-independent (P)RR-mediated intracellular signaling contributes to diabetes-induced retinal leukocyte adhesion. (A) Compared to losartan treatment, PRRB application led to significant ($P < 0.05$) suppression of leukocyte adhesion to the diabetic retinal vessels ($n=10-14$). * $P < 0.05$. (B) 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 ($n=6-8$). * $P < 0.05$, ** $P < 0.01$. Reproduced with permission from (14).

large number of normotensive patients with AMD or 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 (P)RR is present in the major organs but not in the circulation, PRRB does not affect circulatory RAS or

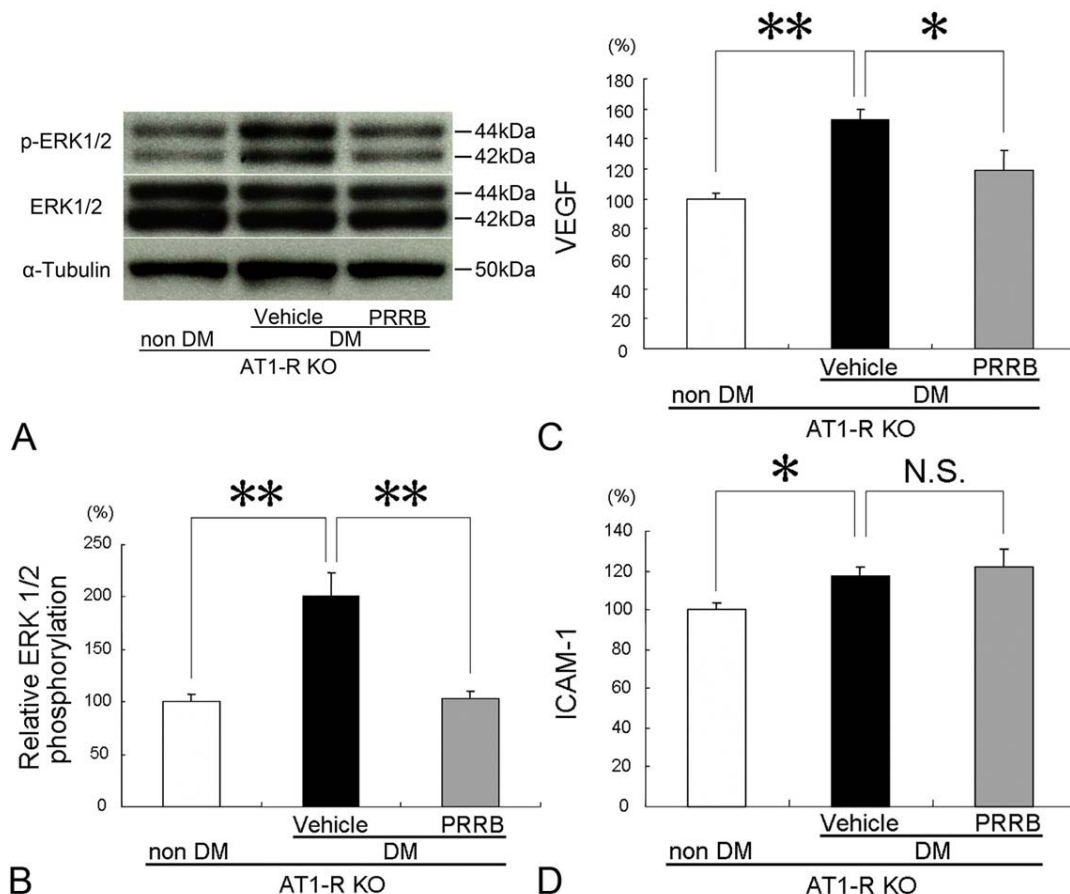


Figure 8. RAS-independent (P)RR-mediated intracellular signaling contributes to ERK1/2 activation and diabetes-induced expression of VEGF, but not ICAM-1 in the diabetic retina. (A-B) Immunoblot analysis for phosphorylated and total levels of ERK1/2 in AT1-R-deficient mice with diabetes. PRRB suppressed relative phosphorylation of ERK1/2 (n=12). Effects by blocking intracellular signaling through (P)RR on protein levels of diabetes-related inflammatory molecules. VEGF levels were significantly suppressed with PRRB (C, n=12-14). ICAM-1 levels showed no significant difference (D, n=12-14). *P < 0.05, **P < 0.01. Reproduced with permission from (14).

systemic blood pressure (7, 8). Interestingly, PRRB treatment to mice with CNV or diabetes-induced retinal inflammation was shown to cause not only tissue RAS deactivation but also additional suppression of (P)RR signaling-mediated expression of MCP-1 and VEGF, the major factor responsible for the development of these angiogenic and inflammatory disorders. Consequently, inhibition of RAPS with PRRB may prove more useful as a novel therapeutic strategy for vision-threatening diseases including AMD and diabetic retinopathy than RAS suppression with conventional AT1-R blockers or angiotensin-converting enzyme inhibitors.

6. ACKNOWLEDGMENTS

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