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REVIEW ARTICLE

(Pro)renin receptor: Involvement in diabetic retinopathy and development of molecular targeted therapy

Atsuhiro Kanda, Susumu Ishida*

Laboratory of Ocular Cell Biology and Visual Science, Department of Ophthalmology, Faculty of Medicine and Graduate School of Medicine, Hokkaido University, Sapporo, Japan

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***Correspondence**
Susumu Ishida
Tel.: +81-11-706-5944
Fax: +81-11-706-5948
E-mail address:
ishidasu@med.hokudai.ac.jp

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ABSTRACT

The renin–angiotensin system (RAS), a crucial regulator of systemic blood pressure (circulatory RAS), plays distinct roles in pathological angiogenesis and inflammation in various organs (tissue RAS), such as diabetic microvascular complications. Using ocular clinical samples and animal disease models, we elucidated molecular mechanisms in which tissue RAS excites the expression of vascular endothelial growth factor (VEGF)-A responsible for retinal inflammation and angiogenesis, the two major pathological events in diabetic retinopathy (DR). Furthermore, we showed the involvement of (pro)renin receptor [(P)RR] in retinal RAS activation and its concurrent intracellular signal transduction (e.g., extracellular signal-regulated kinase); namely, the (P)RR-induced dual pathogenic bioactivity referred to as the receptor-associated prorenin system. Indeed, neovascular endothelial cells in the fibrovascular tissue collected from eyes with proliferative DR were immunoreactive for the receptor-associated prorenin system components including prorenin, (P)RR, phosphorylated extracellular signal-regulated kinase and VEGF-A. Protein levels of soluble (P)RR increased with its positive correlations with prorenin, renin enzymatic activity and VEGF in the vitreous of proliferative DR eyes, suggesting a close link between (P)RR and VEGF-A-driven angiogenic activity. Furthermore, we revealed an unsuspected, PAPS-independent role of (P)RR in glucose-induced oxidative stress. Recently, we developed an innovative single-strand ribonucleic acid interference molecule selectively targeting human and mouse *(P)RR*, and confirmed its efficacy in suppressing diabetes-induced retinal inflammation in mice. Our data using clinical samples and animal models suggested the significant implication of (P)RR in the pathogenesis of DR, and the potential usefulness of the ribonucleic acid interference molecule as a therapeutic agent to attenuate ocular inflammation and angiogenesis.

INTRODUCTION

Diabetic retinopathy (DR), the most common microvascular complication in patients with diabetes, is a leading cause of severe vision loss and blindness in developed countries. Proliferative DR (PDR) is the advanced stage of DR, characterized by proliferation of fibrovascular tissue formed by the extension of retinal angiogenesis into the vitreous cavity, and the fibrovascular tissue formation leads to serious complications including traction retinal detachment and vitreous hemorrhage. A growing body of evidence has accumulated to show that inflammation with leukocyte infiltration plays a crucial role in the pathogenesis of vision-threatening retinal diseases, such as DR[1], which is now considered as an inflammatory as well as angiogenic disease. In acute inflammation, leukocytes infiltrate to extravascular tissues to recognize and exclude the offending agent, mainly contributing to tissue repair. Conversely, leukocytes in chronic inflammation, as seen in diabetes, cause tissue damage as a result of sequential secretion of cytokines, chemical mediators and reactive oxygen species, thus developing a functional maladaptation and tissue remodeling. Inflammatory leakage from the dilated hyperpermeable vasculature leads to the entry of protein- and lipid-containing fluid into the retinal parenchyma, causing frequently observed DR lesions including

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macular edema and hard exudates. Of numerous cytokines and growth factors contributing to the molecular pathogenesis of DR, vascular endothelial growth factor (VEGF)-A has been proven to be the key inflammatory and angiogenic mediator in DR[2–4]. VEGF-A is a secreted cytokine and the most potent angiogenic factor induced mainly by hypoxia- and redox-sensitive transcription factors, and promotes numerous physiological events, such as embryonic development and wound healing. In contrast, VEGF-A is also associated with several pathological events related to various diseases, such as cancer and diabetes. During tumor growth, VEGF-A is predominantly required for new vessel formation, and thus is the major molecular target for anti-angiogenic therapy in cancer[5]. Of several VEGF-A isoforms, we elucidated the significant involvement of VEGF165 with angiogenic activity in PDR, showing that fibrovascular tissues expressing both VEGF receptor-2 and neuropilin-1, VEGF165-specific receptor, were extremely vascularized[1,6,7]. VEGF-A increases the expression of various molecules and subsequently facilitates the infiltration of VEGF-A-releasing leukocytes, which marks a positive feedback loop amplifying the development of retinal inflammation (macular edema) and angiogenesis (fibrovascular proliferation) seen in DR[1,6,7]. In the past decade, therapies targeting VEGF-A have revolutionized the treatment of DR; however, there are several limitations to the therapeutic strategy to inhibit the single molecule in the downstream of a series of pathogenic cascades, and thus an alternative and additive treatment for suppressing some upstream key process at the earlier stages of disease would be desirable as the next-generation management of DR.

The renin–angiotensin system (RAS) was originally considered as a central regulatory mechanism for sodium and fluid homeostasis in controlling systemic blood pressure (i.e., circulatory RAS). Recently, RAS components were also reported to be expressed in numerous tissues independently of circulatory RAS, which is hence called 'tissue RAS'[8]. Tissue RAS works in a paracrine fashion, and controls several physiological and pathological processes including cell signaling and growth, angiogenesis, and tissue remodeling[9–11]. The (pro)renin receptor ([P]RR), which is located in the upstream of tissue RAS, binds with prorenin leading to not only the activation of tissue RAS, but also its intracellular signal transduction, and regulates the expression of various pathogenic molecules including VEGF-A[12]. This dual activation of tissue RAS and RAS-independent signaling pathways through (P)RR is now referred to as the receptor-associated prorenin system (RAPS)[12]. In the present review, we discuss recent progress in the understanding of the significant contribution of (P)RR to the pathogenesis of DR, in combination with our effort to develop an innovative therapeutic agent against (P)RR.

TISSUE RAS IN DIABETIC RETINOPATHY

The initial step of circulatory RAS necessitates the indispensable process of proteolytic activation of prorenin, whereby prorenin is changed to its mature active form; that is, renin by the processing enzymes (e.g., cathepsin B) exclusively in juxtaglomerular cells in the kidney to digest the prorenin prosegment that folds into an active-site cleft of renin. Renin is a rate-limiting enzyme in circulatory RAS for the shedding of angiotensinogen to angiotensin I (Ang I), which angiotensin-converting enzyme changes to angiotensin II (Ang II), the effector molecule that binds to its cognate receptors, Ang II type 1 receptor (AT1R) and Ang II type 2 receptor. By contrast, tissue RAS is characterized by independence from the processing enzyme-based proteolytic activation of prorenin to acquire renin activity, and requires an alternative triggering step caused by (P)RR, as discussed below. Tissue RAS plays several important roles in pathological vascular events, such as angiogenesis and inflammation, and various organ abnormalities are shown to be caused by tissue RAS activation. Regarding its relationship with the eye, multiple clinical trials, such as the EUCLID study, DIRECT-Prevent 1, -Protect 1 and -Protect 2, and the RAS study, showed that inhibition of AT1R or angiotensin-converting enzyme resulted in blood pressure-unrelated beneficial effects on the incidence and progression of DR[13–16]. Ang II levels were reported to significantly increase in the vitreous fluid of PDR eyes, with its significant correlation with VEGF levels[17,18]. Furthermore, we have shown the significant involvement of the AngII/AT1R signaling pathway in inflammation-related ocular angiogenesis, which causes upregulated expression of VEGF-A, C-C chemokine ligand (CCL)2/monocyte chemotactic protein (MCP)-1 and intercellular adhesion molecule-1[19,20], all of which were verified to be responsible for the pathogenesis of DR. These several reports indicate that the activation of tissue RAS in the diabetic eye is the major key event predisposing to the development and deterioration of DR.

(P)RR IN DIABETIC RETINOPATHY

Various organ impairments are reported to result from tissue RAS activation; however, the detailed molecular mechanism for activating tissue RAS still remains unknown. (P)RR, also known as ATP6AP2 (i.e., the gene name for [P]RR) and identified as a single transmembrane protein consisting of 350 amino acids, binds to prorenin to exert renin activity through changes in the three-dimensional structure of prorenin prosegment (i.e., activated prorenin) to expose its enzymatic active-site cleft without the conventional proteolysis of the prorenin prosegment (i.e., renin) achieved by the processing enzymes. This receptor-based non-proteolytic activation of prorenin plays an important role in tissue, but not circulatory, RAS activation, because the membrane-bound (P)RR is shown to exist in various tissues, but not in the circulation[21]. Furthermore, (P)RR interacting with prorenin has been shown to trigger RAS-independent signaling pathways through phosphorylation of extracellular signal-regulated kinase (ERK)1/2[21,22]. Indeed, neovascular endothelial cells of the fibrovascular tissue excised from eyes with PDR were immunopositive for prorenin, (P)RR, phosphorylated ERK1/2 and VEGF-A, in accordance with our *in vitro* data showing

that the prorenin–(P)RR–ERK axis led to an increase in *VEGFA* expression in human retinal microvascular endothelial cells[23]. Thus, we proposed the nomenclature, 'RAPS,' for the (P)RR-induced dual activation of tissue RAS and RAS-independent intracellular signals. (P)RR can interact with both renin and prorenin; however, the binding affinity of renin is much lower than that of prorenin[24], which marks the background of this notation as the '(pro)renin,' but not renin, receptor. RAPS was shown to contribute to the molecular pathogenesis of various ocular disease animal models, such as diabetes-induced retinal inflammation, laser-induced choroidal neovascularization, endotoxin-induced uveitis and oxygen-induced retinopathy[25–28].

VITREOUS RAS AND RETINAL RECEPTOR-ASSOCIATED PRORENIN SYSTEM

Notably, (P)RR was shown to be digested by proteases to turn into a soluble form of (P)RR [s(P)RR]; however, *in vitro*, it still possesses an ability for non-proteolytic activation of prorenin, initiating the conversion of angiotensinogen to Ang I[29]. We have shown that the protein levels of s(P)RR, prorenin, activated prorenin and VEGF-A, in combination with renin activity levels, were remarkably higher in the vitreous fluid samples of PDR eyes than in those of non-diabetic control eyes[23,30]. The vascular density of the fibrovascular tissue and the vitreous protein levels of prorenin, activated prorenin and VEGF-A in PDR eyes were all correlated with elevated levels of s(P)RR generated through shedding of membrane-bound (P)RR from neovascular endothelial cells of the fibrovascular tissue into the vitreous fluid[23]. Importantly, elevated renin activity levels showed positive correlations with the vitreous s(P)RR, prorenin, activated prorenin and VEGF-A protein levels[30]. Our data suggest that the vitreous renin activity stems from s(P)RR-mediated non-proteolytic activation of prorenin, suggesting the important role of (P)RR in the pathogenesis of PDR. RAS components including (P)RR and prorenin were actually detected in human PDR fibrovascular tissues, normal ocular tissues and various human retinal cell lines, such as the retinal pigment epithelium (RPE)[23,31,32], and the vitreous prorenin and Ang II levels were reported to increase in PDR eyes[17,18,23,33]. Furthermore, a close relationship between the vitreous VEGF-A protein and renin activity levels confirmed our concept of 'vitreous RAS' that is involved in the angiogenic activity of DR. Consequently, in concert with retinal RAPS caused by membrane-type (i.e., full-length) (P)RR (Figure 1a)[30], vitreous RAS as a result of s(P)RR[23] (Figure 1b) is presumed to control VEGF-A expression in diabetic eyes.

We have shown that (P)RR signaling through ERK1/2[23,26] and AT1R signaling through nuclear factor (NF)-κB[19] play significant roles in upregulated VEGF-A expression; however, it is challenging to define the ratio of involvement with the angiogenic activity in human PDR. The proprotein convertase, a disintegrin and metalloprotease domain 19[34] and furin[35], processes membrane-bound (P)RR to s(P)RR, both of which were expressed in the neovascular endothelial cells of human PDR fibrovascular tissues[23]. Enzymatic activity and gene expression of these proteases in endothelial cells would likely determine the pathogenic balance between vitreous RAS and retinal RAPS. In the future, research into the physiological and pathological regulation of disintegrin and metalloprotease domain 19 and furin is necessary to reveal RAPS-mediated molecular pathogenesis of DR.

CLINICAL SIGNIFICANCE OF VITREOUS RAS

The importance of the pathogenic system 'vitreous RAS' might lead in part to a possibility of revising the recent surgical indication and concept of vitrectomy for DR. From a clinical point of view, vitrectomy is carried out in PDR eyes for the following reasons: (i) neovessel-derived vitreous hemorrhage that interferes with the visual axis; and (ii) traction retinal detachment in which the retina is elevated by the vitreous that works as the scaffold of the contractile fibrovascular tissue arising from the retina. These two key classic indications to the advanced stage of PDR have long been applied in terms of a mechanical or physical cue. In contrast to this, our findings on vitreous renin activity imply the possibility that the vitreous works as the amplifier of the molecular pathogenesis of DR. Retinal surgeons often see surgical cases, in which diabetic macular edema, caused by VEGF-A-induced vascular hyperpermeability, is reduced soon after vitrectomy. This can be explicated at least in part by the concept of s(P)RR-activated vitreous RAS, the activation of the downstream Ang II/AT1R/NF-κB/VEGF-A pathway causative for the pathogenesis of PDR (Figure 1b). Theoretically, the vitreous might not be just the pool of harmful cytokines, but the generator of pathogenic RAS components. Accordingly, the vitrectomy procedure has a biological sense, which might shift the recent surgical strategy to earlier intervention for broader indications to decrease the vitreous RAS-mediated capability of generating VEGF-A and various other pro-inflammatory and angiogenic cytokines.

SOLUBLE (PRO)RENIN RECEPTOR IN PLASMA OF PATIENTS WITH DIABETIC RETINOPATHY

Besides intraocular environments, s(P)RR levels were elevated in the serum or plasma of patients with several disorders, such as hypertension, obesity, preeclampsia, and kidney and heart failure[36–42]. In the plasma of patients with PDR as well, we showed elevated s(P)RR, renin and activated prorenin protein levels, so as to investigate systemic factors related to plasma levels of these RAS-initiators in patients with PDR[43]. Of several systemic parameters examined, random blood sugar and serum creatinine levels in the PDR patients were significantly higher than those in non-diabetic controls. Plasma s(P)RR levels showed significant correlations with the estimated glomerular filtration rate and serum creatinine, but not prorenin or activated prorenin. Protein levels of tumor necrosis factor (TNF)-α, complement factor D, and leucine-rich α-2-glycoprotein 1 were notably elevated in the plasma of PDR patients as compared with controls. Furthermore, positive correlations were observed between s(P)RR and those inflammation-related molecules, but not prorenin. Excessive glucose during long-standing

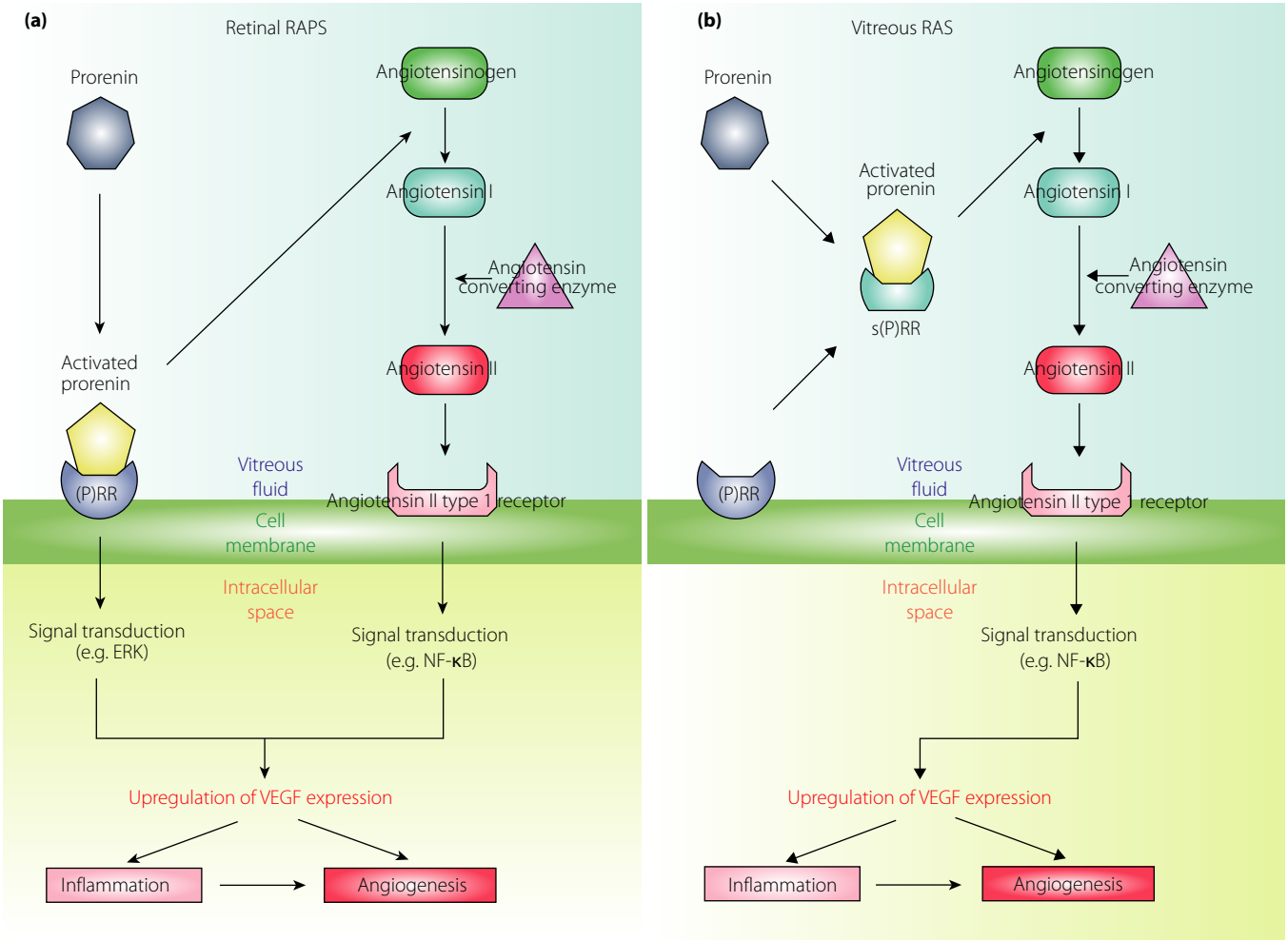

Figure 1 | Molecular mechanisms in retinal (a) receptor-associated prorenin system (RAPS) and (b) vitreous renin–angiotensin system (RAS) leading to vascular endothelial growth factor (VEGF)-driven pathogenesis of diabetic retinopathy. Retinal RAPS is required for membrane-type (pro)renin receptor ([P]RR), whereas vitreous RAS is caused by a soluble form of (P)RR (s[P]RR). Even when membrane-bound (P)RR is truncated into its soluble form, s(P)RR, it continues to affect the pathogenesis driven by angiotensin II signaling. ERK, extracellular signal-regulated kinase; NF-κB, Nuclear factor-κB. Reproduced from Kanda *et al.*[30] with permission.

hyperglycemia in diabetes causes the stimulation of various pathways including advanced glycation end-products formation, the polyol pathway, protein kinase C and p38α mitogen-activated protein kinase activations, and the superoxide pathway[44]. Stimulation of these pathways causes chronic inflammation in numerous tissues, with an increase of inflammation-related molecules including CCL2/MCP-1, intercellular adhesion molecule-1 and TNF-α, all of which were shown to be elevated in the plasma of diabetes patients[44–47]. Complement factor D, a family of serine protease, plays a crucial role in the alternative pathway of complement activation, and mediates chronic inflammation that contributes to diabetic microvascular complications[48–50]. Leucine-rich α-2-glycoprotein 1, a biological marker for several inflammatory disorders, such as asthma, rheumatoid arthritis and ulcerative colitis[51–53], contributes to the formation of pathological neovessels in the retina and tumors[54,55]. Of the inflammation-related molecules correlated with s(P)RR in the PDR plasma, TNF-α, but not complement factor D or leucine-rich α-2-glycoprotein 1, stimulation to human retinal microvascular endothelial cells enhanced the gene expression of (P)RR, but not prorenin, whereas treatment with high glucose upregulated both the messenger ribonucleic acid (RNA) expression of prorenin and (P)RR[43]. Our results showed close links between plasma s(P)RR and diabetes-induced factors, such as chronic inflammation, renal impairment and hyperglycemia in the PDR patients (Figure 2).

(PRO)RENIN RECEPTOR IN GLUCOSE-INDUCED OXIDATIVE STRESS

Recently, we showed an unsuspected function of (P)RR/ATP6AP2 in glucose metabolism[31]. In combination with immunoprecipitation using anti-Atp6ap2 antibody and mass

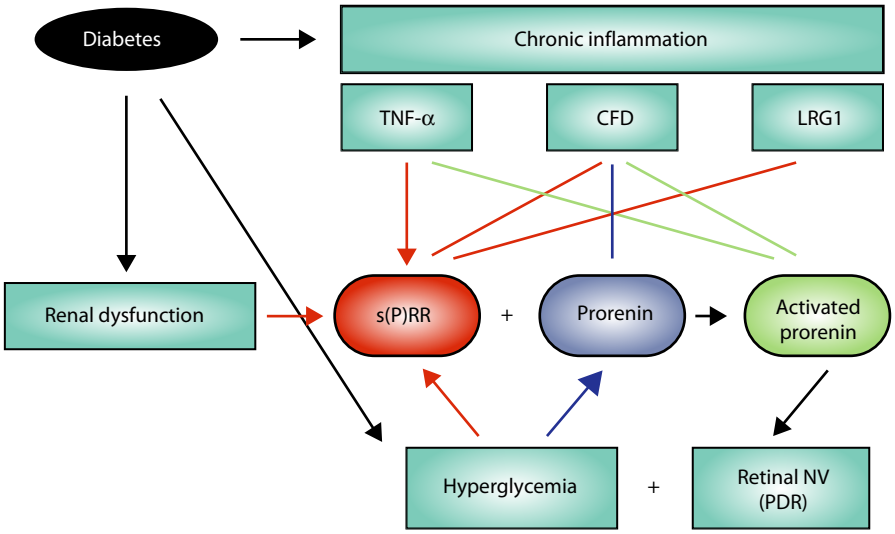

Figure 2 | Association of plasma a soluble form of (pro)renin receptor (s[P]RR) with chronic inflammation, renal dysfunction and hyperglycemia in patients with proliferative diabetic retinopathy (PDR). A schema showing diabetes-induced factors, such as chronic inflammation, renal dysfunction and hyperglycemia, as the potential regulators of plasma s(P)RR and prorenin levels, thus initiating the renin–angiotensin system activation to enhance retinal neovascularization (NV), the hallmark of PDR. Retinal NV, in turn, functions as a cellular source of these RAS initiators under hyperglycemia, generating the vicious cycle of the renin–angiotensin system and PDR. Arrows indicate cause–effect relationships, and lines represent correlations. CFD, complement factor D; LRG1, rich α-2-glycoprotein 1; PDH, pyruvate dehydrogenase; PDHB, pyruvate dehydrogenase E1 β subunit; TNF-α, tumor necrosis factor-α. Reproduced from Hase *et al.*[43] with permission.

spectrometry analyses, we identified pyruvate dehydrogenase (PDH) complex as Atp6ap2-binding proteins in the mature adult mouse retina. Additionally, yeast two-hybrid assays showed direct molecular interaction between ATP6AP2 and the PDH E1 β subunit (PDHB). PDHB is one of the subunits composing PDH complex that changes pyruvate into acetyl-CoA, connecting glycolysis to the tricarboxylic acid cycle and subsequent oxidative phosphorylation. Double labeling experiments showed co-localization of Atp6ap2 with Pdhb in several retinal layers, such as the RPE layer. Depletion of *ATP6AP2* decreased PDH activity, showing a predilection to anaerobic glycolysis in RPE cells. ATP6AP2 was suggested to prevent PDHB being phosphorylated, consequently regulating its protein stability. Decreased PDH activity owing to *ATP6AP2* knockdown repressed glucose-induced oxidative stress in RPE cells. These results revealed the novel biological function of ATP6AP2/(P)RR as a PDHB stabilizer associated with aerobic glucose metabolism and glucose-induced reactive oxygen species production (Figure 3).

Acute and chronic surplus glucose causes devastating alterations in energy metabolism. Under the aerobic condition, elevation of pyruvate, a product of cytosolic glycolysis, facilitates the PDH-mediated conversion of pyruvate to acetyl-CoA followed by mitochondrial nicotinamide adenine dinucleotide-dependent adenosine triphosphate (ATP) synthesis combined with leakage of excess superoxide, causing oxidative stress responsible for the pathogenesis of diabetes[56,57]. Rationally, ATP6AP2 inhibition causes decreased PDH activity, leading to a metabolic shift from aerobic cellular respiration to anaerobic glycolysis together with reduction in mitochondrial oxidative stress. This molecular mechanism is also explained by earlier results showing that PDH was involved in the production of mitochondrial reactive oxygen species and that PDH activity suppression in turn abated oxidative stress[58–60].

The respiratory and circulatory systems, together with efficient energy generation, have been acquired and modified to adapt properly to several environmental changes linked to oxygen concentration during evolution. Energy metabolism is especially active in the vertebrate retina, particularly photoreceptor and RPE cells obtaining ample oxygen supply from the well-developed vasculature of the choroid, which has never been provided for the invertebrate retina with photoreceptors arrayed in front (i.e., back-to-front in comparison with the vertebrate retina). Intriguingly, the amino acid sequence of ATP6AP2 at the specific region binding to PDHB is highly conserved in mammals and fish, but not in *Drosophila*[61], showing that the highly conserved sequence of Atp6ap2 makes it possible to consume high oxygen in the retina by ensuring Pdh enzymatic activity from Pdhb protein degradation. Ironically, as the price for the ATP6AP2-dependent efficient energy generation acquired in human eyes, glucose-induced oxidative stress has emerged as the underlying pathogenesis of DR in the current era of excessive eating.

ATP6AP2/(P)RR plays a crucial role in RAPS activation, which is associated with the molecular pathogenesis of end-organ damage, such as angiogenesis and inflammation seen in

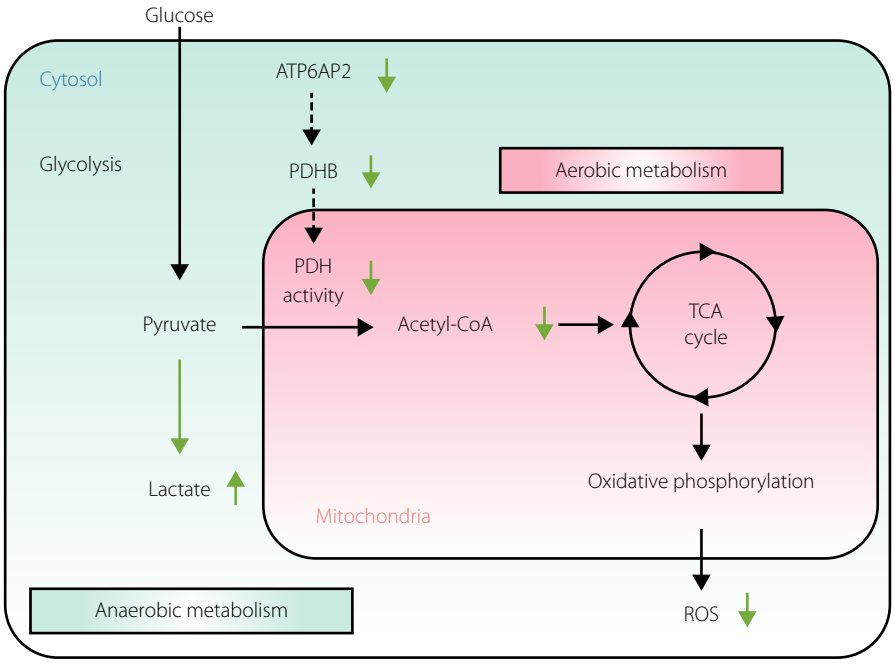

Figure 3 | ATP6AP2/(pro)renin receptor involvement in pyruvate dehydrogenase (PDH)-mediated aerobic glucose metabolism and oxidative stress. ATP6AP2/(pro)renin receptor blockade causes a metabolic shift from aerobic cellular respiration to anaerobic glycolysis (green arrows), leading consequently to a decrease in mitochondrial reactive oxygen species (ROS). Reproduced from Kanda *et al.*[31] with permission. PDHB, pyruvate dehydrogenase E1 β subunit; TCA, tricarboxylic acid.

DR[12,23,26,30]. In concert with our results on its contribution to oxidative stress, ATP6AP2/(P)RR is thought to be pathogenic, with its domain interacting with prorenin and PDHB alike, initiating the distinctly different pathways linked to the molecular pathogenesis of DR (i.e., the activation of retinal RAPS and the generation of mitochondrial reactive oxygen species, respectively). Our new findings on ATP6AP2/(P)RR-PDHB interaction, were also reproduced in a more recent report on hepatic energy metabolism[62].

(PRO)RENIN RECEPTOR IN UVEITIS

In addition to DR, uveitis is also characterized by intraocular inflammation that causes vision loss and blindness after occlusive retinal vasculitis, serous retinal detachment, secondary epiretinal membrane, macular edema and angle-closure glaucoma in some severe cases. To investigate prorenin and s(P)RR involvement in uveitis, we carried out enzyme-linked immunosorbent assay experiments using vitreous aspirates from eyes of patients with uveitis and non-uveitic control eyes with idiopathic epiretinal membrane and macular hole[63]. Notably, RAPS components including prorenin, s(P)RR and activated prorenin significantly increased in the vitreous fluid of uveitic eyes compared with controls. Furthermore, elevated prorenin and s(P)RR levels were significantly correlated with each other. The vitreous levels of cytokines, such as CCL2/MCP-1, interleukin-6, platelet-derived growth factor-BB and VEGF-A, key molecules responsible for neovascularization and inflammation, are known to increase in the eyes of sarcoid uveitis patients[64]. To investigate the relationship between RAPS activation and ocular inflammation, we examined the protein levels of several cytokines in vitreous samples from our uveitic case series. As compared with control eyes, protein levels of CCL2/MCP-1, interleukin-6, platelet-derived growth factor-BB and VEGF-A, but not TNF-α, increased in uveitic eyes. Furthermore, elevated CCL2/MCP-1 levels were significantly correlated with increased RAPS parameters, including (s)PRR, prorenin and activated prorenin. These results show that the close link between the RAPS components and CCL2/MCP-1 levels would validate the pathogenic role of (P)RR that contributes to inflammation in human uveitis. Furthermore, we have shown that RAPS activation contributes to the molecular pathogenesis, including inflammation, angiogenesis and fibrosis, of other ocular disorders, such as conjunctival lymphoma[65] and idiopathic epiretinal membrane[66].

(PRO)RENIN RECEPTOR IN RETINAL DEVELOPMENT

(P)RR was initially identified as a cleaved form of (P)RR working as a subunit of vacuolar H^+-ATPase (v-ATPase), an ATP-dependent multi-subunit proton pump, and termed ATP6 accessory protein 2 (Atp6ap2)[21,67]. The v-ATPase plays important roles in numerous physiological and fundamental cellular activities, such as endocytosis, processing of proteins, and the activation of lysosomal and autophagosomal enzymes[68]. Atp6ap2/(P)RR and v-ATPase-mediated acidification were

recently reported to be important for both of the Wnt/β-catenin and Wnt/planar cell polarity signaling pathways in *Drosophila* and *Xenopus*[69–71]. Cardiomyocyte- or podocyte-specific gene deletion in mice showed that Atp6ap2 is necessary for the maintenance of cell structure and survival[72–74]. Consequently, Atp6ap2/(P)RR is proposed to be associated not only with tissue RAS activation, but with various physiological processes through the v-ATPase function.

Although our data using clinical samples showed that ATP6AP2/(P)RR contributes to angiogenic activity in human PDR eyes[23,30], there was no report showing functional analysis or the physiological role of ATP6AP2/(P)RR in the mammalian retina. Furthermore, to avoid unexpected adverse effects in clinical trials, it is necessary to understand the physiological function of a target molecule. Given that (P)RR is one of embryonic-essential genes[75], as well as VEGF-A[76], we generated photoreceptor (i.e., rod and cone)-specific conditional knockout mice using the Cre-LoxP system, so as to show the essential role of Atp6ap2/(P)RR in photoreceptor development[32]. The absence of photoreceptor *Atp6ap2/(P)RR* did not cause significant changes in retinal cell differentiation, but in disorganization of laminar formation in the outer nuclear layer combined with critically impaired photoreceptor cell alignment. Cell polarity and adhesion proteins co-localized with Atp6ap2/(P)RR at the apical edge of the normally developing retina; however, these molecules were markedly interspersed and retinal progenitor cells mis-localized from the apical surface in *Atp6ap2/(P)RR*-conditional knock-out mice. Of cell polarity and adhesion proteins, we identified that Atp6ap2/(P)RR bound to partitioning defective 3 homolog (PAR3) protein with co-immunoprecipitation using mouse retinal homogenates and *ATP6AP2/(P)RR*-transfected human embryonic kidney 293T cells. The Par family proteins contribute to forming cell polarity, controlling cellular asymmetry through distribution and formation of molecular complexes[77]. Par3 makes a complex with atypical protein kinase C (aPKC)λ and Par6 (i.e., the Par–

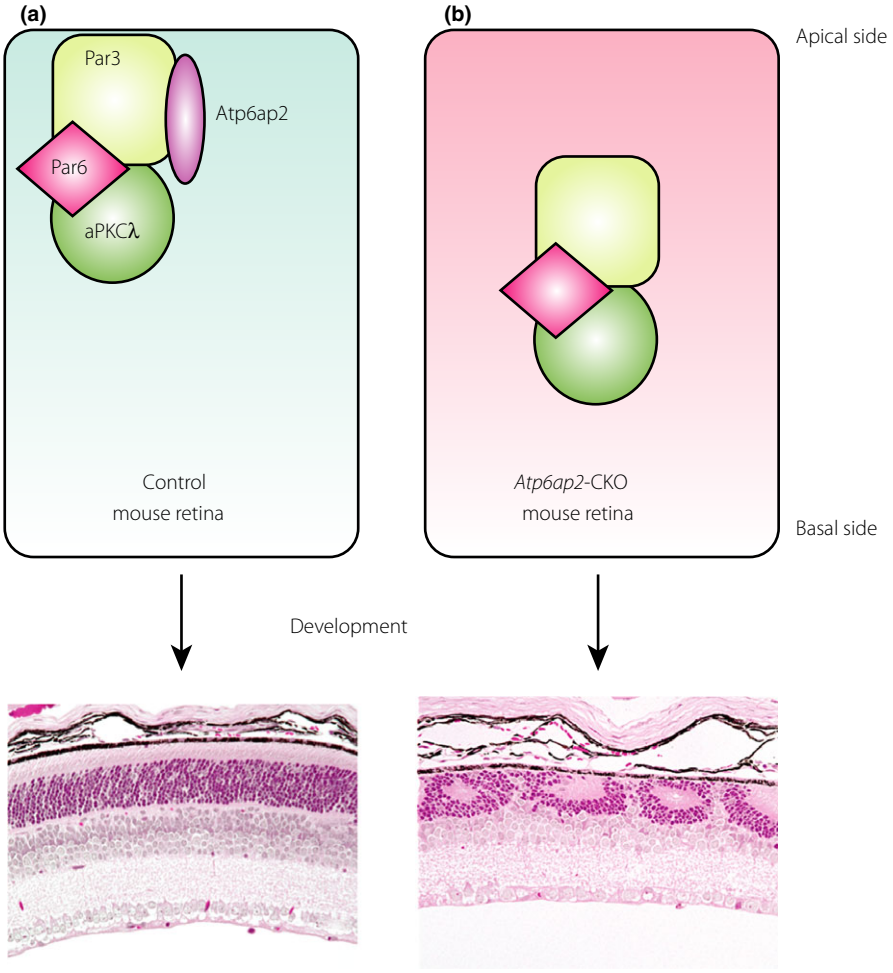

Figure 4 | Impaired retinal development in photoreceptor-specific *Atp6ap2*-deficient mouse. (a) Normal and (b) photoreceptor-specific *Atp6ap2* deficient mouse retina. Atp6ap2/(pro)renin receptor binds to partitioning defective 3 homolog (Par3) as a cell polarity determinant required for retinal laminar organization during physiological development. Modified from Kanda *et al.*[32] with permission. aPKCλ, atypical protein kinase Cλ.

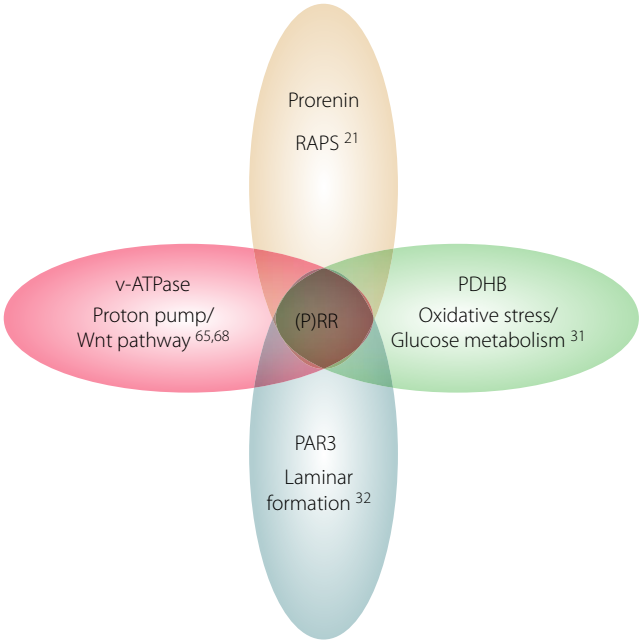

Figure 5 | Binding partners and biological functions of (pro)renin receptor ([P]RR)/ATP6AP2. (P)RR/ATP6AP2 interacts with various molecules to exert distinctly different functions. PAR3, partitioning defective 3 homolog; PDHB, pyruvate dehydrogenase E1 β subunit; RAPS, receptor-associated prorenin system; v-ATPase, vacuolar-type H^+-adenosine triphosphatase.

atypical protein kinase C system), specifically located on cellular junctions, and is involved in the cell polarity regulation in various cells[78]. Furthermore, direct molecular binding between ATP6AP2/(P)RR and PAR3 was observed in yeast two-hybrid assays. Our findings showed the new physiological function of Atp6ap2/(P)RR during retinal development involved in laminar formation (Figure 4).

We suggest that this novel cellular activity of ATP6AP2/(P)RR contributing to the Par–atypical protein kinase C system, besides the v-ATPase function, the activation of tissue RAS and the stabilization of PDHB protein, is the fourth biological role of Atp6ap2/(P)RR in retinal laminar formation (Figure 5). However, this physiological role of (P)RR during development would not be a hurdle for our inhibition of (P)RR for the purpose of treating DR and other inflammatory diseases, such as uveitis, because the pathological role of (P)RR as the RAPS activator is basically seen in the adult stage.

DEVELOPMENT OF A NOVEL SINGLE-STRAND RNA INTERFERENCE THERAPEUTIC AGENT TARGETING (PRO)RENIN RECEPTOR

Based on our findings[23,25–28,30,43,65,66], a blockade of (P)RR is theorized to inhibit the cascade of events crucial in various vascular abnormalities represented by inflammation and angiogenesis. Aliskiren, a direct renin inhibitor, competitively inhibited the renin enzymatic activity of both renin and activated prorenin through interaction with (P)RR *in vitro*; however, RAS inhibitors including aliskiren have no efficacy of blocking (P)RR's own downstream signals[29]. (P)RR blocker, also known as handle-region peptide, is a peptide with the structure of the handle region of the prorenin prosegment working as a decoy for (P)RR. At present, (P)RR blocker is the only available agent to inhibit prorenin-(P)RR interaction (i.e., acquisition of renin enzymatic activity and transduction of receptor signaling) subsequently leading to RAPS activation[79]. We and others have shown that (P)RR blocker potently suppressed the pathogenesis of various disease models in target organs (e.g., DR, diabetic nephropathy, neovascular age-related macular degeneration, uveitis and retinopathy of prematurity)[22,23,25–27]. However, a blockade of ligand-receptor protein interaction using decoy peptides has several limitations: (i) requirement of an excessive amount of peptides; (ii) induction of immune response and autoantibodies; (iii) protease resistance; and (iv) high molecular weight. These inherent and serous issues with decoy peptides are likely to preclude future clinical application.

To block the pathological function of (P)RR, we designed a new class of RNA interference (RNAi) agent, proline-modified short hairpin RNA (PshRNA), to knockdown human and mouse *(P)RR* mRNA (Figure 6). RNAi is basically considered

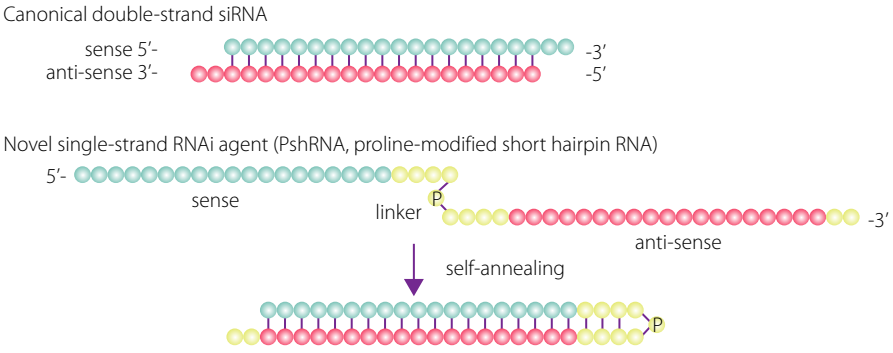

Figure 6 | Structure of proline-modified short hairpin ribonucleic acid (PshRNA). Structure of canonical double-strand small interfering RNA (siRNA) and novel single-strand RNA interference (PshRNA) agents. Blue circles indicate the sense strand of a target gene, red circles are the antisense strand and yellow circles are the linker region. P indicates a proline derivative. RNAi, ribonucleic acid interference.

to have minimal cellular toxicity due to its endogenous cellular function for controlling gene expression, and thus contains attractive and promising aspects for the development of new therapies. With regard to clinical application, however, there are some obstacles that need to be overcome. In general, canonical double-strand small interfering RNAs (siRNAs) are recognized by family members of the toll-like receptor (TLR) and retinoic acid-inducible gene-I-like receptor, causing the activation of intracellular signaling pathways to initiate innate immunity[80]. Recently, a single-strand RNAi has proved to be capable of sequence-specific gene silencing through the RNAi system without off-target expression of inflammatory cytokines via TLR-mediated signal transduction in rodent eyes[81]. Further, although an annealing process is required for the generation of a double-strand siRNA, it is not necessary for a single-strand RNAi because the linker is replaced with proline derivatives and can therefore self-anneal. Previous studies showed that PshRNAs were more stable against nucleases than canonical double-strand siRNAs[82]. Taken together, the single-strand RNAi strategy appears to overcome some of the drawbacks faced by canonical double-strand siRNAs.

To design PshRNA against (P)RR ([P]RR-PshRNA), we first carried out *in silico* analysis regarding various parameters, such as length, structure, sequence, and chemical and nucleotide compositions, all of which mediate efficient RNAi, and generated several candidate RNAi agents targeting a different nucleotide sequence of *(P)RR* gene common to both species. We then tested their knockdown efficiency in preliminary experiments using human and mouse cell lines, so as to select one candidate as (P)RR-PshRNA in terms of both potency and persistency of *(P)RR* knockdown. Real-time reverse transcription polymerase chain reaction and immunoblot analyses showed that the levels of *(P)RR/ATP6AP2* transcript and product significantly decreased after exposure to human RPE and mouse endothelial cells with (P)RR-PshRNA as well as (P)RR-siRNA in a dose-dependent manner. To determine tissue distribution of (P)RR-PshRNA injected into the vitreous cavity of murine eyes, we used tetramethylrhodamine-labeled (P)RR-PshRNA. The labeled (P)RR-PshRNA signals were deeply penetrated and widely distributed to the ganglion cell layer, inner and outer nuclear layers, and RPE in the posterior segment of the eye, and to the corneal epithelium and stroma in the anterior segment of the eye. Importantly, the newly designed (P)RR-PshRNA showed more robust nuclease resistance than the conventional double-strand (P)RR-siRNA, and did not affect retinal function and structure.

The endotoxin-induced uveitis model is frequently used as an acute inflammation model in various organs including the eye, and streptozotocin-induced diabetes is a type 1 diabetes model due to impaired insulin secretion from pancreatic β-cells injured by streptozotocin toxicity. Given that DR has recently been regarded as an inflammatory disorder, we used these models to examine the effect of (P)RR-PshRNA on acute and chronic inflammation. Previously, we reported the significant suppression of intraocular inflammation in this model by blocking AT1R and (P)RR to inhibit tissue RAS and RAPS, respectively[19,26]. (P)RR-PshRNA application to mice caused significant amelioration of acute (uveitic) and chronic (diabetic) models of ocular inflammation; that is, the total number of retinal adherent leukocytes, and upregulated gene expression levels of *Il-6*, *Ccl2/Mcp-1*, *Icam-1*, *Tnf-a* and *(P)RR/Atp6ap2*, as seen in endotoxin-induced uveitis and streptozotocin-induced diabetes mice treated with phosphate-buffered saline or control-PshRNA, were significantly suppressed with administration of (P)RR-PshRNA[63]. As we described above, using conditional knockout mice, we have revealed that (P)RR/Atp6ap2 contributes to physiologically essential cellular functions that are independent of RAPS; however, we did not observe any adverse events after application with (P)RR-PshRNA *in vivo* and *in vitro*. It is possible that the *in vivo* knockdown at the dose is too weak to cause any side-effects, despite its significant efficacy in suppressing ocular inflammation. Importantly, the currently designed sequence for RNAi targeting *(P)RR* is common to human and mouse genes, indicating that the multimodal animal testing with (P)RR-PshRNA would also serve as a useful reference for human clinical trials.

CONCLUSION

Our results might lead to a novel understanding of the molecular mechanism in pathological events including glucose-induced oxidative stress, vascular inflammation and retinal angiogenesis, all of which are regulated by (P)RR. VEGF, the key modulator of DR, is dually governed by retinal RAPS and vitreous RAS, either of which is triggered by (P)RR and s(P)RR, respectively. Our ongoing development of (P)RR-PshRNA, an innovative single-strand RNAi agent targeting (P)RR, will soon promote clinical research on several eye diseases, especially DR, thus aiming at further improvement of visual prognosis in DR patients.

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DISCLOSURE

The authors declare no conflict of interest.

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