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REVIEW

Signal transduction of the (pro)renin receptor as a novel therapeutic target for preventing end-organ damage

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The (pro)renin receptor ((P)RR) not only represents a novel component of the renin–angiotensin system but is also a promising novel drug target because of its crucial involvement in the pathogenesis of renal and cardiac end-organ damage. This review discusses the signal transduction of the (P)RR with its adapter protein promyelocytic zinc-finger protein, the impact of this receptor, especially on cardiovascular disease, and its putative interaction with renin inhibitors such as aliskiren. Furthermore, the increasing complexity regarding the cellular function of the (P)RR is addressed, which arises by the intimate link with proton pumps and the phosphatase PRL-1, as well as by the presence of different subcellular localizations and of a soluble isoform of the (P)RR. Finally, the rationale and strategy for the development of small-molecule antagonists of the (P)RR, called renin/prorenin receptor blockers, are presented.
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INTRODUCTION

Renin and prorenin are classically thought of as (pro)enzymes, but recent evidence suggests that they also can act as hormones because of their ability to bind cellular targets.[1] A human (pro)renin receptor ((P)RR)—also termed renin/prorenin receptor (RER)—which can specifically bind prorenin and renin, has been cloned, consisting of 350 amino acids with a single transmembrane domain.[2] Interestingly, this receptor—which is expressed in organs such as the brain, heart, kidney, liver and pancreas[2,3]—was reported to exert a dual function:[2,3]

(1) Binding of renin to this receptor increases the catalytic activity of renin by about four- to fivefold. Furthermore, prorenin, which does not exhibit significant ability to generate angiotensin I (ang I) in solution, gains enzyme activity comparable to that of renin by binding to the (P)RR, that is, the receptor is able to unmask the catalytic activity of prorenin.
(2) The (P)RR is also able to induce a signal-transduction cascade upon ligand binding. Binding of renin and also prorenin causes a phosphorylation of the receptor and a modest activation of the MAP (mitogen-activated protein) kinases (MAPKs), ERK1 and ERK2, whereas intracellular calcium or cAMP levels are not altered.

SIGNAL TRANSDUCTION OF THE (P)RR

As no direct protein interaction partner was described in the initial characterization of (P)RR[2] and because protein–protein interactions are crucial in understanding the mechanisms of a signal transduction cascade, our group analyzed protein–protein and downstream protein–DNA interactions of the (P)RR. Initially, we found an intracellular and ubiquitous expression pattern of the human (P)RR.[4] Consistent with the latter and with housekeeping gene properties, we observed several transcriptional start sites within the TATA box-less human (P)RR promoter and a high promoter activity in different cell types.[4]

By yeast two-hybrid screening and co-immunoprecipitation, we identified promyelocytic zinc-finger (PLZF) protein as a direct protein interaction partner of the C-terminal domain of the (P)RR. PLZF is a zinc-finger transcription factor that is disrupted in patients with translocation t(11;17)(q23;q21)-associated acute promyelocytic leukemia.[5]

Co-immunoprecipitation experiments also indicated homodimerization of the (P)RR.[4] On activation of the (P)RR by renin, PLZF is translocated into the nucleus and represses transcription of the (P)RR itself, thereby creating a very short negative feedback loop, but activates transcription of the p85α subunit of the phosphatidylinositol-3 kinase (PI3K-p85α) (Figure 1). Short-interfering RNA (siRNA)

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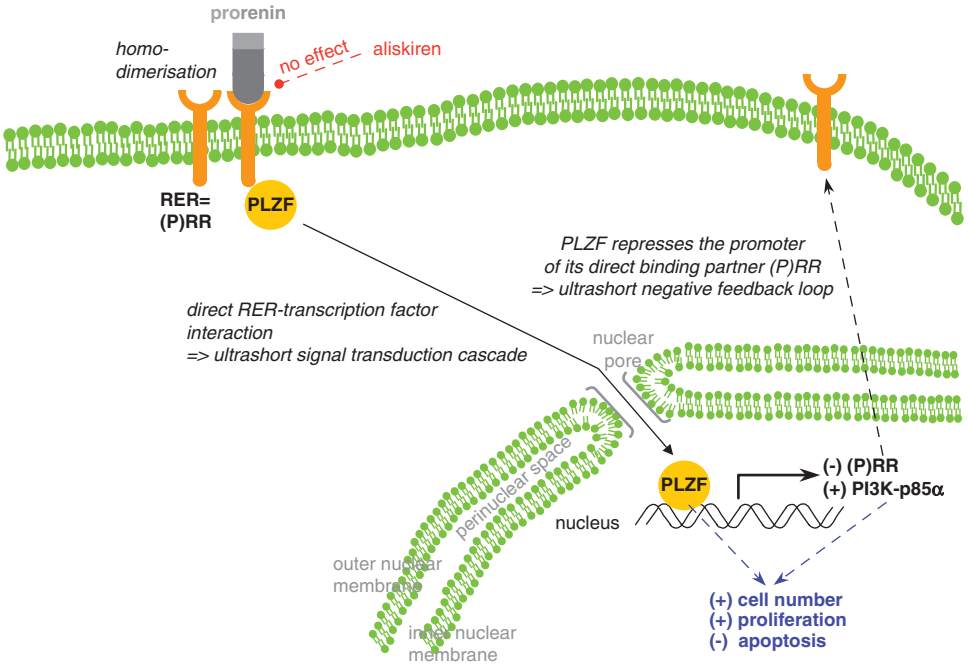

Figure 1 Signal transduction of the (P)RR based on protein–protein and protein–DNA interactions.

against the (P)RR abolished these effects. A PLZF *cis*-element in the (P)RR promoter was identified by site-directed mutagenesis and electromobility shift assay. In addition, renin stimulation caused a sixfold recruitment of PLZF to this promoter region, as shown by chromatin-immunoprecipitation.[4] Moreover, renin stimulation of rat H9c2 cardiomyoblasts induced an increase in cell number and a decrease in apoptosis. These effects were partly abolished by PI3K inhibition and completely abrogated by siRNA against PLZF.[4] Consistently, PI3K-p85α and PLZF are known to be involved in stimulation of protein synthesis and cardiac hypertrophy.[6,7] Finally, experiments in PLZF knockout mice confirmed the role of PLZF as an upstream regulator of (P)RR and PI3K-p85α.[4]

Recently, we demonstrated that, besides renin, prorenin also induces the (P)RR-PLZF-PI3K-p85α pathway[8] (Figure 1). Furthermore, prorenin exerts proproliferative and antiapoptotic effects to a similar extent as renin, which are fully mediated by (P)RR and PLZF, as shown by siRNA experiments. Remarkably, the novel renin inhibitor (RI) aliskiren does not interfere with the intrinsic activity, that is, the noncatalytic effects, of both renin and prorenin.[8]

These data demonstrate the existence of a novel signal transduction pathway involving the ligands renin or prorenin the (P)RR and the transcription factor PLZF, which is involved in cellular proliferation and apoptosis (Figure 1).

Besides the recruitment of PLZF, other groups observed the activation of MAPKs downstream of the (P)RR.[2,9] This MAPK activation after stimulation of the (P)RR with renin occurs relatively late, with a maximum described after 30 min,[2] compared with ang II stimulation experiments that peak at about 5 min.[10] In addition, Akt kinase, which itself is located downstream of PI3K, can activate MAP kinases by mechanisms involving, for example, protein kinase C and nitric oxide[11,12] (Figure 2). Therefore, we speculate that PLZF acts upstream of MAP kinases (Figure 2), which is supported by a recent publication that reports that expression of PLZF can enhance the activity of ERK kinase.[13] Nevertheless, further experiments involving, for example, tandem affinity purification, chromatin-immunoprecipitation and siRNA are necessary to address the putative link between PLZF and MAPK. Very recently, it was demonstrated that both renin and prorenin can induce MAPKs in renal MDCK cells in the presence of ang AT1 receptor (AT1R) and ang AT2 receptor blockade.[14] Nevertheless, these effects were only marginally affected by siRNA against the (P)RR, implicating other receptors in the regulation of MAPKs by (pro)renin.

Consistent with our *in vitro* results, the downregulation of (P)RR mRNA by high levels of renin was also observed by other groups in certain animal models. In healthy normotensive rats, treatment with a vasopeptidase inhibitor decreased blood pressure (BP), increased renal renin mRNA and decreased (P)RR mRNA to about 80%.[15] Furthermore, administration of an angiotensin-converting enzyme (ACE) inhibitor, in addition to a low-salt diet, increased renin mRNA and protein but decreased renal (P)RR mRNA, as expected from our *in vitro* results.[16] Consistently, inhibition of prorenin binding to the (P)RR by the use of the handle region peptide, which itself is discussed below, increased retinal (P)RR mRNA.[17]

In contrast, in the Goldblatt two kidney-one clip model, BP reduction increased plasma prorenin, plasma renin and renal renin mRNA expression, and also caused a parallel increase in (P)RR mRNA expression in the clipped kidney.[15]

In this regulatory context, it is of interest to note that diabetic rats exhibit an approximately twofold increase in renal (P)RR expression on mRNA and protein levels compared with normal rats.[18] Interestingly, valsartan prevented this increase by interfering with the AT1R and the downstream NADPH oxidase.[18]

BIOMEDICAL RELEVANCE OF THE (P)RR

Developmental biology

It has already been shown in 2005 that a monogenic defect in the gene encoding the (P)RR can be the cause for X-linked mental retardation associated with epilepsy in humans.[9] Consistent with this observation,

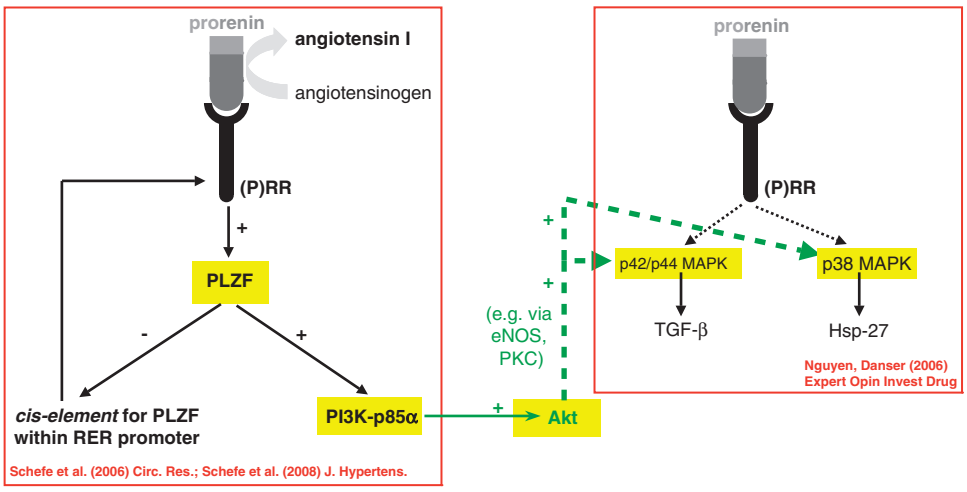

Figure 2 Signal transduction of the (P)RR—putative link between PLZF and MAPK.

the gene encoding (P)RR has a crucial role in zebrafish brain development.[19] In addition, the (P)RR seems essential for early murine embryonic development, as embryonic stem cells with a mutation in the (P)RR gene are incompatible with the development of chimeric mice when injected into blastocysts.[20] Therefore, the generation of (P)RR knockout mice has not been possible so far.

Cardiovascular diseases

Cardiac, renal and ophthalmological end-organ damage due to hypertension and/or diabetes is currently one of the major medical challenges.[21,22] They contribute to approximately 60–80% of all heart failure[23,24] and to 70% of renal failure[25,26] cases. In addition, about 30% of blindness in Western countries is caused by these diseases.[27–29] Current therapeutic strategies, such as ACE inhibitors, ang AT1R blockers, β-adrenergic receptor antagonists or antidiabetic agents only ameliorate but do not fully prevent or abolish cardiovascular end-organ damages.[30–32]

The relevance of the (P)RR to these conditions is underlined by the impressive observations of Ichihara's group, which show that a decoy peptide corresponding to the handle region of prorenin named handle region peptide, which competitively inhibits prorenin binding to its receptor, attenuated the development and progression of hypertension-induced cardiac fibrosis,[33,34] and also completely inhibited the development of diabetic nephropathy in different rat models including AT1R knockout mice.[32,35] Consistently, it was shown that (P)RR activation can induce transforming growth factor-β1 in mesangial cells.[36] Furthermore, transgenic overexpression of the (P)RR in smooth muscle cells causes a BP elevation and an increase in heart rate,[37] whereas an ubiquitous transgenic overexpression accounts for glomerulosclerosis and proteinuria.[38] Other groups were unable to confirm the effects of the decoy peptide *in vitro* or *in vivo*;[39–41] however, questions on handle region peptide degradation and bioavailability have not been addressed in greater detail in these publications. Another recently proposed hypothesis to explain the discrepancies with respect to the beneficial effects of the prorenin-derived decoys implicates the renin level, as the decoys were only effective in low-renin and not in high-renin animal models.[42–44] In this context, it is important to note that the decoys competitively inhibit not only the binding of prorenin but, unexpectedly, also of renin to the (P)RR.[45]

In favor of Ichihara's handle region peptide, an independent group recently demonstrated that the decoy peptide reduced left ventricular mass index, proteinuria and creatinine in salt-overloaded spontaneously hypertensive rats.[46] Furthermore, the (P)RR blocking decoy peptide was shown to abolish the renin effect on the action potential frequency in neuronal cells *in vitro*.[47] Finally, ischemia-initiated retinal neovascularization,[48] as well as diabetes-induced retinal inflammation,[49] can be reduced significantly by prorenin-derived decoys.

Regarding the (P)RR ligand prorenin, a plethora of publications have addressed its effect on the development of end-organ damage in transgenic animal models. Liver-targeted overexpression of rat prorenin caused severe renal damage (for example, glomerulosclerosis) and myocardial hypertrophy in the absence of hypertension and without an alteration in plasma renin activity compared with non-transgenic rats.[50] In contrast, an inducible, transgenic, hepatic prorenin overexpression using indole-3-carbinol was associated with hypertension, moderate renal vasculopathy and moderate cardiac hypertrophy, but not with cardiac fibrosis and glomerulosclerosis,[51,52] whereas a similar transgenic experiment observed glomerular damage.[53] A recent, sophisticated approach used transgenic mice expressing site-mutated prorenin eliminating its enzymatic activity.[54] These animals did not exhibit cardiac fibrosis or renal glomerular sclerosis. Consistently, a further transgenic model with a high-plasma wild-type prorenin level showed only modest renal lesions and myocardial fibrosis at 6 months.[55] In double-transgenic mice overexpressing prorenin and angiotensinogen, end-organ damage was not analyzed in greater detail, but major abnormalities in heart or kidney using routine histological staining were not reported.[56]

Despite these controversies and complexities of animal models, plasma prorenin levels are predictors of the consecutive development of diabetic nephropathy and retinopathy in the human species.[57,58]

Pharmacology—RIs

The (P)RR has gained increasing interest in the pharmaceutical industry because of the development of RIs with oral activity, such as Ro 66–1132 (Hoffmann-La Roche, Basel, Switzerland) or Aliskiren (CGP 60536B, SPP-100; Novartis, Basel, Switzerland).[59–61] As expected, RIs reduce plasma renin activity (that is, enzyme activity with respect to ang I generation) and by this action, plasma levels of ang I and ang II as well. Nevertheless, the total amount of plasma

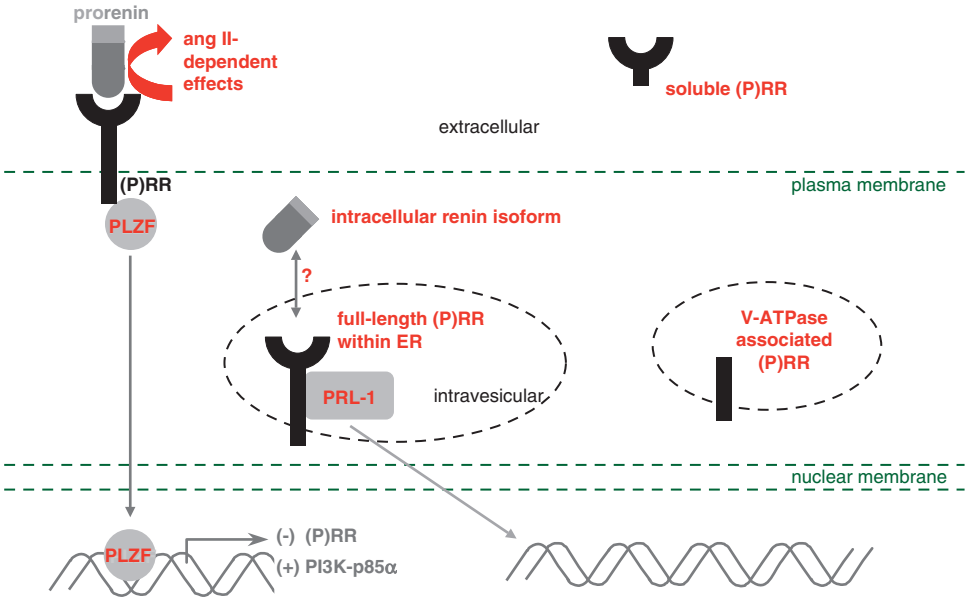

Figure 3 Increasing complexity of (P)RR-mediated cellular effects.

renin is dramatically increased on RI treatment (up to 34-fold[62]) because the negative feedback of ang II on renin is interrupted.[59,63,64] The 34-fold increase might be overestimated because of an assay artefact: by binding to prorenin, aliskiren causes a conformational change in this molecule, which is then detected as (immuno-re)active renin, that is, aliskiren allows the false detection of prorenin as renin.[65–67] Nevertheless, renin inhibition can cause a greater increase in plasma renin compared with AT1R blockade at dosages with comparable antihypertensive effects.[68] It has been reported that the plasma renin concentration under 300 mg aliskiren is almost doubled compared with 320 mg valsartan.[43] In this context, studies by Laragh should be mentioned, which demonstrated that the reactive increase in renin secretion can even limit the effectiveness of aliskiren in sub-populations of patients with highly reactive renin levels, that is, the therapeutic aliskiren concentration is relatively too low to compensate for the increase in renin.[69,70] In these studies reanalyzed of Laragh's group, BP did not decrease in most low-renin patients and even increased in 5% of patients taking aliskiren. In contrast, a very recent reexamination of three aliskiren trials questions the methodology used by Laragh and observed—using a different stratification cutoff—that no patient with a medium-to-high plasma renin activity at baseline had an increase in both plasma renin activity and BP.[71] This implies that no treatment failure is present in this sub-population that is related to a 'disturbed stoichiometry' between increased renin and aliskiren. Nevertheless, even this study detected 'a few' patients—but not those with an increment in plasma renin activity—in whom BP increased.

Unfortunately, to our knowledge, studies directly comparing the relative effects of ACE inhibitor, ang AT1R blockers and aliskiren on plasma prorenin and renin levels in parallel—as well as the associated antihypertensive response rates—are missing. Regarding the origin of plasma renin, it is important to note that in healthy subjects and in those with essential hypertension, the prorenin plasma concentration is about ninefold higher than the renin concentration.[72,73] Furthermore, at extrarenal sites—which are not subjected to the classical negative feedback loop on renin release mediated by renal AT1Rs—only prorenin is synthesized.[74] These extrarenal sites contribute to 30–40% of the total plasma prorenin.[44]

In conclusion, it is likely that RIs will indirectly affect the activity of (P)RR *in vivo*. As discussed above[8] and also shown by other groups,[67,75] aliskiren does not inhibit the ability of renin and prorenin to induce a signal-transduction cascade at the (P)RR *in vitro*.

Preliminary data of our group involving 12 individuals indicate that a 3-week aliskiren therapy—compared with the pretreatment state—can decrease (in 2 of 12 cases) and also increase (in 2 of 12 cases), or does not alter (in 8 of 12 cases), the (P)RR mRNA expression in blood cells of humans *in vivo*. On the basis of our *in vitro* results[4] and our expression analysis of PLZF knockout mice,[4] especially the increase is unexpected. Therefore, factors such as cell type, species, *in vitro*–*in vivo* transferability, signal transduction kinetics, environment, age, gender and/or genetic variabilities might affect the regulation of the (P)RR. Consistent with the latter (that is, genetic subgroups), several single nucleotide polymorphisms have been recently described in the human (P)RR gene.[76] Among these, the promoter polymorphism −782A>G was weakly associated with ambulatory BP. The intronic single nucleotide polymorphism 169C>T was associated with a 24 h systolic BP difference of 6.4 mmHg in men which even exceeds the effect of the well-known ACE I/D polymorphism.

Oncology

(P)RR mRNA can be detected in human glioblastomas and in glioblastoma cell lines in which RIs reduce the cell number. This reduction is probably caused by a modulation of (P)RR function, as this effect is independent of AT1R and AT2R activity.[77] The direct protein–protein interaction of the (P)RR with the oncogenic protein phosphatase of regenerating liver-1 (PRL-1), which is discussed below, also illustrates a putative role of this receptor in oncology.

INCREASING COMPLEXITY

The (P)RR does not exhibit a homology to any other known receptor family.[2] Nevertheless, the C-terminal 69–100 amino acids of the (P)RR are identical to the vacuolar proton-translocating ATPase (V-ATPase or vacuolar H^+-ATPase) membrane sector-associated protein M8-9 (APT6M8-9, also known as ATP6AP2 or ATP6M8-9; GenBank

identifier number GI:5031590)[14,78] (Figure 3). V-ATPases, which are ATP-dependent proton pumps, are involved in several cellular functions such as neurotransmitter uptake and storage, endocytosis, receptor recycling and urinary acidification.[14,79] The V-ATPase-associated part of the (P)RR—in contrast to the (pro)renin binding domain—is evolutionarily conserved, as it shows a high sequence homology between vertebrates (for example, mammals, fish) and invertebrates (for example, *Caenorhabditis elegans* and *Drosophila*).[80] Consistently, the ligand renin is only expressed in mammalian and—as recently demonstrated in zebrafish—nonmammalian vertebrates.[81] Angiotensinogen, the substrate of renin, is also expressed in zebrafish.[82]

It was shown by different groups that full-length (P)RR is mainly localized intracellularly in perinuclear compartments, most likely corresponding to the endoplasmic reticulum.[4,83] Interestingly, transient transfections of different (P)RR expression constructs—each C- and also N-terminally fused to EGFP—indicated that the V-ATPase segment of the (P)RR showed a different localization pattern compared with the full-length (P)RR, as it was localized primarily to the lysosomal compartment.[4] The largely intracellular location is also supported by the observation that (P)RR and CAPER (*Homo sapiens* endoplasmic reticulum-localized type I transmembrane adaptor precursor) are identical transcripts (GenBank accession number AY038990). CAPER was identified by yeast two-hybrid screening using the ubiquitous tyrosine phosphatase PRL-1 as bait (personal communication). The protein–protein interaction of (P)RR and PRL-1 was confirmed by co-immunoprecipitation (Schefe JH *et al*., unpublished data) (Figure 3). PRL-1 is involved in the regulation of cellular proliferation, transformation, as well as in tumor formation in nude mice, and exhibits a cell cycle-dependent subcellular localization.[84,85] It is present in the endoplasmic reticulum in resting cells but is localized to centrosomes and to the spindle apparatus in mitotic cells. The role of PRL-1 with regard to the cellular effects of the renin–angiotensin system is currently unknown.

The preferential intracellular presence of the (P)RR contrasts with the cell surface (that is, plasma membrane) localization initially described by Nguyen *et al*.,[2] but this discrepancy might be explained by cell-type differences. Consistent with this view, preponderant (P)RR protein expression was observed at the luminal surface of rat kidney collecting duct intercalated cells.[14]

The coupling of (P)RR to MAP kinases and PLZF, as well as the extracellular nature of the ligands prorenin and renin, indicates that the signal transduction of this receptor is initiated at the plasma membrane, where about 10% of the (P)RR protein can be detected.[20] However, other scenarios are also feasible. It has been hypothesized by Michael Bader that a further receptor for a soluble (pro)renin receptor might exist.[80] This soluble form of the (P)RR termed s(P)RR with a molecular weight of 28 kDa was recently described in the medium of different cell types, as well as in rat and human plasma.[86] Regarding its biosynthesis, the group of Nguyen confirmed the presence of full-length (P)RR in the endoplasmic reticulum. After trafficking to the Golgi apparatus, full-length (P)RR is cleaved in the *trans*-Golgi by the action of furin generating s(P)RR and the transmembrane-cytoplasmic domain[86,87] (Figure 3). The latter likely corresponds to the V-ATPase segment of the (P)RR and is retrieved in some lysosomes, whereas noncleaved (that is, furin escaped) full-length (P)RR is addressed to the plasma membrane.[86,87]

In this context of intracellular (P)RRs, it is important to note that, besides the 'classical' preprorenin mRNA, a second mRNA isoform encoding a nonsecreted (that is, intracellular) form of active renin has been cloned in the rat, mouse and human species. This new isoform contains an alternative first exon that was termed—although identical—'exon 1b'[88,89] and 'exon 1A'.[90,91] 'Renin b' ('exon 1A renin' or 'exon(1A-9)renin'[92]) is expressed in the brain and in adrenocortical mitochondria. Remarkably, it is the only renin transcript in the heart—in contrast to the cardiac uptake of renin and prorenin proteins[91]—where it is also upregulated in infarction.[93] Exon(1A-9)renin encodes a truncated, cytosolic prorenin, as it lacks the prefragment of secretory renin and the first 10 amino acids of the prosegment.[92] Recently, it was shown that this cytosolic renin isoform can mediate antinecrotic but proapoptotic effects in cardiomyocytes.[92] Nevertheless, a recent knockout model indicates that intracellular renin cannot functionally compensate for a loss of secreted renin.[94]

Despite this pathophysiological relevance, a rendezvous between this nonsecreted, cytoplasmic renin and (P)RR is unlikely, because the (pro)renin-binding domain of the latter is localized intravesicularly, that is, not directly in the cytoplasm, or extracellularly[80] (Figure 3).

THE DEVELOPMENT OF RERBS

On the basis of its crucial role in the pathophysiology of renal and cardiac end-organ damage, the (P)RR is a promising novel therapeutic drug target for cardiovascular disease. Furthermore, cardiovascular end-organ damage represents an unmet medical need, because current therapeutic strategies, as mentioned above, only ameliorate but do not abolish this life-threatening condition. Regarding small-molecule drug development, high-throughput screening assays represent a classical first step toward exploring the chemical space for pharmacological activity.[95] Therefore, the (pro)renin-(P)RR-PLZF-RER/PI3K pathway discussed above was filed as a patent (EP 1 890 152 A1), as it can be used as a readout for (P)RR activity within a high-throughput screening assay.

The importance of this pathway in mediating end-organ damage is also demonstrated by the observation that left ventricular hypertrophy is absent in PLZF knockout mice subjected to ang II infusion.[96] Furthermore, the nuclear translocation of PLZF downstream of an AT2 receptor activation was associated with an increased protein synthesis and with growth stimulation of cardiomyocytes.[6] Furthermore, PLZF is a candidate gene for obesity and insulin sensitivity,[97] and an upstream regulator of the renal epithelial sodium channel.[98]

Supported by the GO-Bio initiative of the German Federal Ministry of Education and Research (BMBF), the aim of an interdisciplinary project team at the Center for Cardiovascular Research (CCR)/ Institute of Pharmacology of the Charité in Berlin is to develop a novel drug class called RER blockers (RERBs). RERBs will represent small molecules with oral bioavailability and the ability to inhibit the RER for the indication of hypertension- and diabetes-related renal and cardiac end-organ damage. In theory, RERBs should block both the ang II-dependent (that is, enhancement of (pro)renin's catalytic activity) and ang II-independent (that is, intrinsic activity of renin and prorenin) effects of the (P)RR.

A high-throughput screening assay based on stably transfected cell lines and a luciferase readout will be performed to screen approximately 100 000 compounds of a library for inhibitory effects on the RER in cooperation with a contract research organization. To validate the putative confirmed compounds generated by this primary screening, a secondary screening based on nuclear translocation of PLZF upon (pro)renin stimulation will be used to yield the so-called hits. The methods for a subsequent hit-to-lead program to filter down these multiple hits according to pharmacodynamic, pharmacokinetic and toxicological parameters are currently being established. In addition,

a medicinal chemistry program to optimize hits with appropriate pharmacological parameters will be run.

The *in vivo* proof-of-concept, which tests the effects of lead compounds with RERB activity in animal models of renal and cardiac end-organ damage, constitutes the final milestone within our BMBF GO-Bio project.

Renin/prorenin receptor blockers not only represent future drugs for human clinical trials but also experimental tools to shed light on the fascinating and complex world of the (P)RR and its adapter proteins.

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