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HRP and prorenin: focus on the (pro)renin receptor and vacuolar H^+-ATPase

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1. ABSTRACT

The function of prorenin, the precursor of renin, remained unknown until the discovery of the (pro)renin receptor ((P)RR). (Pro)renin binding to this receptor allows angiotensin generation and induces signaling. Thus, (P)RR blockade will exert effects beyond angiotensin suppression. Recently, the (P)RR has been identified as a subunit of the vacuolar-type H^+-ATPase, with important roles in Wnt signaling. In addition, transgenic animals overexpressing prorenin display the consequences of angiotensin generation, whereas transgenic animals overexpressing the (P)RR display an angiotensin-independent phenotype. Finally, both beneficial and deleterious effects have been described following treatment with the (P)RR antagonist 'handle region peptide' (HRP), while a (P)RR knockout in cardiomyocytes is lethal. This review highlights the latest findings in the (P)RR area, focusing on cardiovascular and renal pathology. It critically addresses the possibility that (pro)renin acts as an agonist of this receptor *in vivo*, and discusses the efficacy of HRP. Conclusions are that convincing evidence for (pro)renin-(P)RR interaction *in vivo* is currently lacking and, thus, that the concept of HRP exerting beneficial effects by blocking such interaction remains to be proven.

2. INTRODUCTION

The renin-angiotensin system (RAS) has a well-established function in blood pressure regulation and body fluid homeostasis. Renin catalyzes the hydrolysis of angiotensinogen into angiotensin (Ang) I, which is further metabolized by angiotensin-converting enzyme (ACE) into the vasoactive peptide Ang II. Renin is formed from an inactive precursor, prorenin, by cleavage of a 43-amino acid aminoterminal prosegment exclusively in the juxtaglomerular cells of the kidney and secreted into the circulation. Prorenin is secreted constitutively, mainly from the kidney, but also to a lesser extent from other organs, including the reproductive organs, eye and adrenal glands (1). Plasma prorenin concentrations are higher, sometimes up to 100-fold, than plasma renin concentrations (2). Early findings indicated a correlation between elevated plasma prorenin concentrations and the incidence of macro- and microvascular complications, such as nephropathy and retinopathy, in patients with diabetes mellitus (3, 4), even though active renin concentrations were normal or reduced. Since RAS activity is not restricted to the circulation but also present in tissues that are targets of end-organ damage in cardiovascular disease (5-10), prorenin seemed a likely contributor to tissue angiotensin production. However,

proteolytic activation of prorenin could never be demonstrated outside juxtaglomerular cells. The cloning and characterization in 2002 (11) of a (pro)renin receptor ((P)RR) that acts both as an activator of and signaling receptor for prorenin seemed to provide an elegant solution to this problem and a novel potential target to treat cardiovascular and renal complications. Now, nearly a decade later, the picture is far from clear to what extent the (P)RR contributes to end-organ damage, and whether this involves Ang II-dependent mechanisms. In addition, novel functions for the (P)RR have been identified as a subunit of the vacuolar-type H^+-ATPase, with important roles in Wnt signaling, independent from binding of (pro)renin. This review highlights the latest findings on the multiple functions of the (P)RR, and provides a critical assessment of the role of the (P)RR in cardiovascular and renal pathology and the efficacy of the putative (P)RR antagonist HRP.

3. (P)RR: MORE THAN JUST A (PRO)RENIN RECEPTOR?

The (P)RR is a 350-amino acid protein that can bind both renin and prorenin (11-15). The (P)RR induces a conformational change in prorenin, by which the prosegment is moved out of the catalytic cleft and the active site is exposed leading to full, non-proteolytic activation of prorenin (11-14). Surprisingly, the (P)RR also directly activates signaling pathways, independent from the formation of Ang II. Binding of (pro)renin to the (P)RR activates the mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase 1/2 (Erk1/2) in several cell types, including vascular smooth muscle cells (VSMCs), mesangial cells, monocytes, collecting duct cells, neurons, endothelial cells and adipocytes (16-24). Activation of the Erk1/2 pathway results in up-regulation of profibrotic genes, including transforming growth factor-β_1 (TGF-β_1), plasminogen activator inhibitor-1 (PAI-1), fibronectin and collagen-1, and increased cell proliferation (18, 25, 26). Binding of (pro)renin to the (P)RR also activates a p38 MAPK-Heat shock protein 27 (Hsp27) cascade that regulates actin cytoskeletal dynamics in cardiomyocytes (27), and induces the nuclear translocation of promyelocytic leukaemia zinc finger (PLZF) protein, which activates the expression of the p85-subunit of phosphoinositide 3-kinase (PI3K), resulting in enhanced protein synthesis, cell proliferation and decreased apoptosis (15, 28).

A recent series of papers have shown that the (P)RR also has important functions in embryonic development and signal transduction, independent from binding of (pro)renin. In fact, homology studies have shown that the C-terminal domain of the (P)RR is highly conserved in all metazoans, while the extracellular domain is conserved in vertebrates only, indicating that (pro)renin binding could be a function that was derived later during vertebrate evolution (29). Although the (P)RR was reported to have no homology to any known proteins when it was first cloned (11), it was later found that the C-terminal domain of the (P)RR is identical to the 8.9 kDa accessory protein ATP6AP2 of the vacuolar H^+-ATPase (30). Vacuolar H^+-ATPases (V-ATPases) are multisubunit complexes composed of two domains, with V_1 carrying out ATP hydrolysis and V_O forming the proton channel. V-ATPases are expressed in virtually all cell types and found mainly on the membranes of intracellular compartments. They play an important role in the acidification of subcellular compartments, such as endosomes, Golgi apparatus, and lysosomes, thus facilitating receptor mediated endocytosis, protein trafficking and protein degradation (31, 32). In some cell types, V-ATPases are also abundantly present at the plasma membrane and have specialized functions, for example urine acidification in collecting duct cells, bone resorption in osteoclasts, and cell migration and angiogenesis in endothelial cells (33-36). In addition, V-ATPases have been implicated in tumor cell invasion and virus entry into cells (37-39). Genetic defects in patients and in animal models in components of the V-ATPase have underscored the importance of the V-ATPase in physiology and embryonic developments. Mutations in several subunits affect embryonic development and are either embryonic lethal, or result in severe phenotypes, including distal renal tubular acidosis, osteopetrosis and sensorineural deafness (33, 40-43).

Mutations in the (P)RR result in comparable developmental defects. In zebrafish insertional mutations in the *(P)RR* gene as well as genes encoding other V-ATPase subunits are embryonic lethal, resulting in smaller heads and eyes, underdeveloped liver and guts, necrosis in the central nervous system and lack of pigmentation (44, 45). In *Xenopus*, injection of morpholino antisense RNA against (P)RR in cleavage-stage embryos interferes with gastrulation and results in phenotypes similar to zebrafish (P)RR mutants, with tadpoles that have small heads, shortened tails and defects in melanocyte and eye pigmentation (46, 47). Also in higher vertebrates, the (P)RR seems essential for embryonic survival, as attempts to generate conventional (P)RR deficient mice have so far not yielded any viable offspring. Because of this, genetic models to study the function of the (P)RR in cellular and organ physiology are so far lacking. However, patients from a single family that have a splicing mutation in the *(P)RR* gene resulting in 50% truncated (P)RR protein have X-linked mental retardation and epilepsy, indicating important functions in the regulation of brain development and neuron biology (48). Further, a recent study by Kinouchi *et al* (49) showed that in mice, cardiomyocyte specific ablation of the *(P)RR* gene resulted in lethal heart failure, due to deacidification of intracellular vesicles and extensive vacuolization of cardiomyocytes, elucidating for the first time that the (P)RR is indispensable for the mammalian V-ATPase.

Intriguingly, the (P)RR was identified in both *Drosophila* and *Xenopus* in genome-wide siRNA screens as a novel component of the Wnt/β-catenin signaling pathway. The Wnt family of signaling proteins are important regulators of antero-posterior axis formation and patterning. They are also involved in adult tissue homeostasis, and aberrant Wnt signaling has been implicated in diseases, including cancer (50-52). Wnt proteins signal through a complex consisting of frizzled

(Fz) receptors that are heterodimerized with low-density lipoprotein receptor-related proteins (LRPs). In the canonical Wnt pathway, Wnt binding results in stabilization of β-catenin, which then translocates to the nucleus where it complexes with the T cell factor/lymphocyte enhancer-binding factor (TCF/LEF) to induce the expression of Wnt target genes. (P)RR knock-down in *Xenopus* and *Drosophila* reduces canonical Wnt signaling, as Wnt-target gene expression is reduced and wing development in *Drosophila* disturbed (46, 47, 53). Wnt can also activate other signaling pathways, for example the planar cell polarity (PCP) pathway, which polarizes cells in the plane of tissue (54). PCP also requires the (P)RR, as (P)RR depletion causes gastrulation defects in *Xenopus* (47) and misorientation of wing hairs and notum bristles in *Drosophila* (47, 53).

What is the role of the (P)RR in Wnt-signaling? Consistent with an important function in different Wnt pathways, the (P)RR acts as a physical adaptor between the Fz-receptor complex (46, 47, 53) and the V-ATPase complex (46), and in *Drosophila* is required for the asymmetrical distribution of Fz-receptors in PCP (53). Both Wnt-induced LRP6 phosphorylation, which is required for Wnt-signaling (55), and β-catenin dependent gene expression are impaired when the (P)RR or other V-ATPase components are knocked down or when cells are treated with the selective V-ATPase inhibitors bafilomycin or apicularen A (46). Furthermore, direct tagging of LRP6 with the pH-sensitive green fluorescent protein pHluorin showed that LRP6 is incorporated in acidic vesicles after Wnt-stimulation, which is blocked by apicularen (46). These findings suggest an important function for the (P)RR as an adaptor between the Fz-LRP6 complex and the V-ATPase during endocytosis and subsequent acidification of signaling endosomes in Wnt-signaling (Figure 1, left panel). The concept of the V-ATPase as a regulator of signal output is not exclusive for Wnt signaling, as for example also trafficking of the Notch complex in *Drosophila* is dependent on the V-ATPase (56). Similarly, other pH-regulating proteins can regulate signaling, as PCP in *Drosophila* also requires activity of the sodium-proton exchanger NHE2 (57), in agreement with a possible function for protons as mediators of signal transduction pathways (58).

Since the (P)RR is an essential component of the V-ATPase complex (49), is there a function for the V-ATPase in prorenin-induced signaling? A recent paper by Advani *et al.* (20) seems to suggest so. The authors found that the (P)RR colocalizes with the V-ATPase at the plasma membrane in the intercalated cells of the collecting duct. In Madin-Darby canine kidney (MDCK) epithelial cells, (pro)renin induced Erk1/2 activation that was attenuated both in (P)RR knock-down cells and by the selective V-ATPase inhibitor bafilomycin. These findings strongly suggest a function for the V-ATPase in signaling through the (pro)renin-(P)RR axis. The mechanism by which the (P)RR signals to downstream effectors is as yet poorly understood, but some putative mechanisms are suggested in Figure 1 (right panel). The (P)RR has no homology to known signaling receptors or obvious protein-protein interaction domains (11). PLZF has been identified as a binding partner for the (P)RR, but is a transcriptional regulator of gene expression rather than a signaling intermediate (15, 28). So far, studies have focused on the consequences of (P)RR knock-down and V-ATPase inhibition on signaling, but the direct effects of (pro)renin on V-ATPase activity are still unknown. At least in cardiomyocytes and embryonic fibroblasts, the (P)RR is required for the stability of the V_O domain of the V-ATPase, as knocking down the (P)RR decreases protein levels of V_O, but not V_1 subunits (49). Thus, the (P)RR might simply be required to keep the complex together, and (pro)renin could further stabilize the complex or induce conformational changes that stimulate proton translocation (mechanism 1). Another possibility is that (pro)renin binding to the (P)RR alters V-ATPase trafficking. In renal proximal tubule cells, Ang II stimulation of V-ATPase activity involves activation of the p38 MAPK (59). Since p38 MAPK is also an intermediate for (pro)renin in the regulation of the actin cytoskeleton (27) and V-ATPase trafficking depends on actin cytoskeletal dynamics, (pro)renin binding could lead to increased trafficking of V-ATPase-containing vesicles to the plasma membrane (60) (mechanism 2). How proton translocation regulates signaling downstream of the (P)RR is as yet unknown. Increases in cytosolic pH are a permissive signal for certain signaling pathways, for example phosphorylation of Erk1/2 by Ang II in rat aorta smooth muscle cells (61), and likewise increased V-ATPase activity at the plasma membrane could stimulate Erk1/2 phosphorylation. As a third possible mechanism, the (P)RR could play a facilitating role in receptor activation in signaling endosomes (43). Many G-protein coupled receptors (GPCRs), including AT_1 receptors, require internalization for proper signaling (62). This is facilitated by β-arrestins, that in turn act as scaffolds for multiple signaling proteins, including Erk1/2, and under certain conditions can even do so in a ligand-independent fashion (63). Studies from vascular smooth muscle cells that express the human (P)RR suggest that the (P)RR cycles between intracellular compartments and the plasma membrane (12). Possibly, (pro)renin binding to the (P)RR allows these systems to come together in endosomes and/or increases vesicular acidification by the V-ATPase, which could then somehow trigger signaling by GPCR-associated β-arrestin scaffolds (mechanism 3). These models are of course still hypothetical and need experimental data, not the least the effect of (pro)renin on V-ATPase localization and activity.

4. (P)RR AND PRORENIN IN PATHOLOGY

Is there a function for prorenin and the (P)RR in cardiovascular complications? (P)RR expression is upregulated under several pathological conditions, for example in the heart and kidney of rats with congestive heart failure (64), in the heart of stroke-prone spontaneously hypertensive rats (SHRsp) on a high salt diet (65), in kidneys of rats with end-stage diabetic nephropathy (66, 67), in remnant kidneys of nephrectomized rats (68), and in the clipped kidney of Goldblatt hypertensive rats (69). Under glycemic conditions, (P)RR expression is upregulated by signaling cascades involving protein kinase

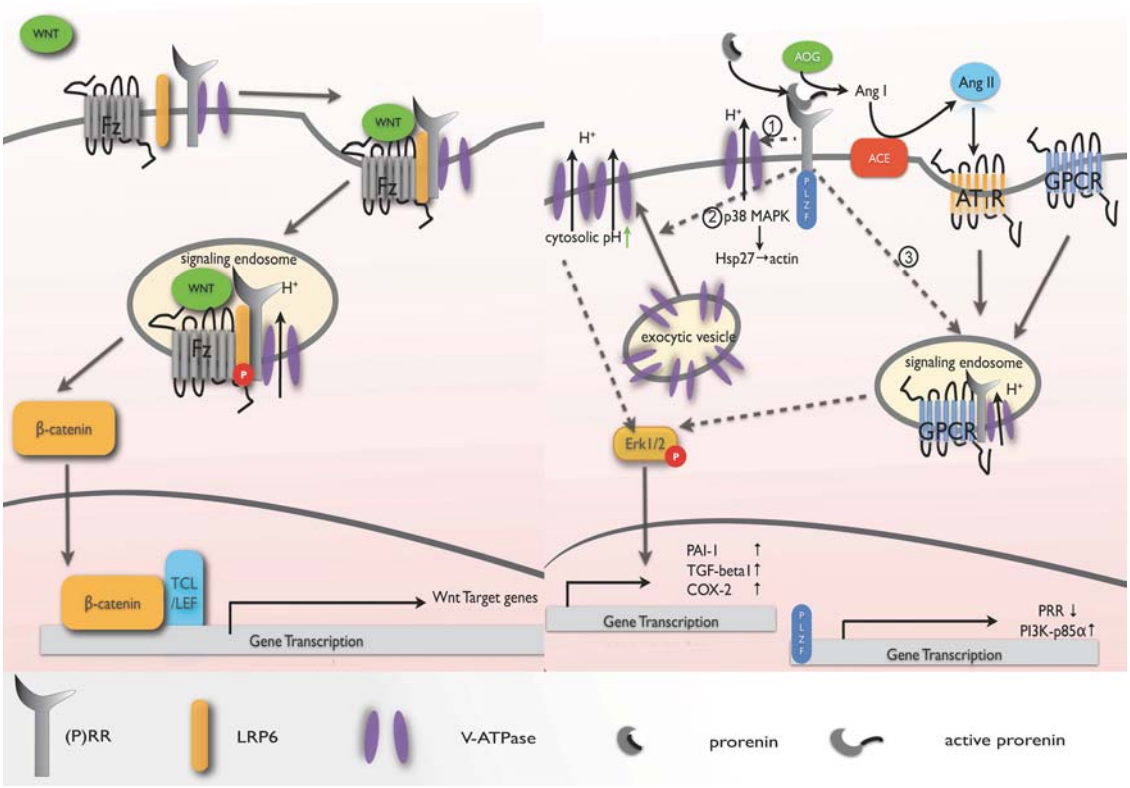

Figure 1. Schematic representation of the (P)RR and the V-ATPase in signal transduction pathways. Left panel: (P)RR in canonical Wnt signaling. Right panel: putative mechanisms of the (P)RR in prorenin initiated signal transduction. See text for details. Abbreviations used: Ang, angiotensin; Aog, angiotensinogen; ACE, angiotensin-converting enzyme; AT1R, Ang II type 1 receptor; COX-2, cyclooxygenase-2; Erk1/2, extracellular signal-regulated kinase 1/2; Fz, frizzled; GPCR, G-protein coupled receptor; Hsp27, heat-shock protein 27; p38 MAPK, p38 mitogen activated protein kinase; PAI-1, plasminogen-activator inhibitor-1; PI3K, phosphoinositide 3-kinase; PLZF, promyelocytic leukaemia zinc finger protein; (P)RR, (pro)renin receptor; TCF/LEF, T cell factor/lymphocyte enhancer-binding factor; TGF-β_1, transforming growth factor-β_1

C activation of MAP kinases and nuclear factors NF-κB and AP-1 (70), and induces expression of the proinflammatory factors interleukin-1β and cyclooxygenase-2 (71). *In vivo* studies using transgenic rats that overexpress the human (P)RR support the concept of Ang II-independent effects induced by the (P)RR (72, 73). When expressed in cultured cells, the human (P)RR binds, but does not activate rat prorenin, which implies that observed effects in human (P)RR transgenic rats are due to direct signaling through the (P)RR (73). These rats have an elevated blood pressure and increased aldosterone levels (72), and showed a progressive development of proteinuria and glomerulosclerosis without an increase in renal Ang II levels (73). Furthermore, in agreement with studies in mesangial cells, MAPK phosphorylation and TGF-β_1 expression was increased in the kidneys of these rats (73).

Experiments using an inhibitory peptide called the handle region peptide (HRP), which binds competitively to the (P)RR (discussed below), seem to corroborate a function for prorenin in end-organ damage. Infusion of HRP decreases cardiac Ang II levels and attenuates cardiac fibrosis in SHRsp rats (65), normalizes renal Ang II levels, inhibits the development of diabetic nephropathy in diabetic mice (74) and attenuates ischemia-induced retinal vascularization in mice (75), indicating a role for the (P)RR in the pathologic tissue RAS. However, HRP also reduced glomerulosclerosis in diabetic mice that are deficient for the Ang II type 1a receptor (AT_{1a}) (76), which no longer respond to ACE inhibition. Moreover, HRP, but not the ACE inhibitor imidapril, inhibited glomerulosclerosis and proteinuria in human (P)RR transgenic rats (73). This indicates that some effects of (P)RR activation are independent of tissue generation of Ang II. Others, however, found no effects of HRP on blood pressure, cardiac hypertrophy and renal damage when infused in 2-kidney 1-clip Goldblatt rats (77). Furthermore, as will be discussed later, HRP did not prevent signaling by (pro)renin in monocytes (19) or vascular smooth muscle cells (16).

Studies with transgenic rats and mice that overexpress prorenin also yielded conflicting results. In an earlier study, Véniant *et al.* (78) found that rats with constitutive hepatic expression of prorenin, resulting in 400-fold increased circulating prorenin levels, developed cardiac hypertrophy and renal lesions. These rats did not develop hypertension, which raised the possibility that high prorenin concentrations lead to tissue damage independent of the generation of Ang II. Subsequent work, however,

contradicted these findings. In a study of 2009, the same rats did develop hypertension, resulting in only modest renal injury and cardiac fibrosis late in life (79). Glomerulosclerosis also did not occur in transgenic rats with inducible prorenin expression, despite the fact that such rats, following induction, displayed a 200-fold rise in plasma prorenin and were hypertensive (80). In addition, transgenic mice with 13-66 fold higher concentrations of circulating prorenin have increased blood pressure, but do not develop cardiac fibrosis or glomerulosclerosis (81). Importantly, the ACE inhibitor captopril normalized blood pressure in the latter mice, and no hypertension was observed in mice that expressed prorenin with an active site mutation. Collectively, these results indicate that the primary effects of prorenin overexpression are due to Ang II-dependent hypertension (82).

The increase in plasma prorenin levels in the above transgenic animals is much higher than observed in diabetic patients, where increases are <5-fold (3). Plasma prorenin levels are also increased under other normal or clinical conditions, but to date there are no clinical data to support a role for prorenin in end-organ damage. For instance during normal pregnancy, plasma prorenin levels are elevated without cardiovascular or renal manifestations (83). The renin inhibitor aliskiren, like other RAS blockers, causes an increase in renin due to a lack of negative feedback by Ang II on renin release (84). Simultaneously, a modest increase in prorenin levels occurs (85). Aliskiren does not interfere with (P)RR signaling (86), but does inhibit the activity of (P)RR-bound-(pro)renin (13). Yet, no clinical study has ever found complications in patients that could be linked to excess prorenin levels during RAS blockade. One explanation could be that (pro)renin binding to the (P)RR induces nuclear translocation of the transcription factor PLZF, where it inhibits expression of the *(P)RR* gene itself, (28), thus establishing a negative feedback loop that prevents overactivation of (P)RR-induced signaling. This is supported by the fact that (P)RR expression is decreased in diabetic rats overexpressing the murine *renin2* gene when treated with aliskiren (86). In apparent contrast with this observation, increased plasma (pro)renin and renal renin levels coincided with upregulated (P)RR expression in the clipped kidneys of Goldblatt rats (69). Thus, the regulation of (P)RR expression is complex and depends on more factors than just prorenin levels alone.

Other unresolved issues are the localization and ligand affinity of the (P)RR. The carboxyterminal side of the (P)RR contains two conserved putative targeting domains for endosomal and lysosomal sorting and ER retention (29), and concomitantly the majority of the (P)RR protein is present in intracellular compartments, with only a small portion present at the plasma membrane (27). The binding affinity of the (P)RR for prorenin and renin is in the nanomolar range, as opposed to the levels of circulating endogenous prorenin, which are in the low picomolar range in normal subjects (2). In addition, in most studies signaling responses are only evident when cells are stimulated with nanomolar concentrations of prorenin. Exceptions are mesangial and collecting duct cells, in which picomolar concentrations of (pro)renin are sufficient to induce Erk1/2 phosphorylation (18, 20). In the collecting duct, the (P)RR is mainly present at the plasma membrane of the intercalated cells (20). Since the collecting duct is a major source of prorenin in diabetes (87), it is possible that sufficient receptor occupancy can only be reached in tissues where prorenin is produced and the (P)RR abundantly present at the plasma membrane.

In summary, (pro)renin can act *in vitro* as an agonist of the (P)RR to directly activate signaling cascades, however, evidence that this also occurs *in vivo* is inconclusive. Instead, studies using transgenic animals that overexpress prorenin suggest that the predominant function of the (P)RR appears local, non-proteolytic activation of prorenin facilitating the generation of Ang II at tissue sites (79, 81, 82). Since the (P)RR has prorenin-independent function as well in V-ATPase assembly (49) and Wnt-signaling (46, 47, 53), the effect of (P)RR overexpression in pathogenic and transgenic models could also be explained by overactivation of other signaling pathways. Indeed, aberrant Wnt signaling has been implicated in diabetic retinopathy (88), and in renal diseases, including renal fibrosis and diabetic nephropathy (89-92).

5. HRP: A (P)RR ANTAGONIST?

As discussed, renin and prorenin bind with nanomolar affinity to the (P)RR, K_D's ranging from ≈1-20 nM depending on the use of immobilized receptors or membrane fractions of (P)RR-transfected cells (11, 12, 14, 93-96). When comparing renin and prorenin in the same assay, most studies revealed that the K_D value of prorenin for the human (P)RR is 3-4-fold lower than that of renin. Therefore, it seems reasonable to assume that the prosegment facilitates binding. A peptidic antagonist has been designed based on the idea that the prosegment of prorenin contains a 'handle region' (10P-19P) which binds to the receptor, allowing prorenin to become catalytically active (97). This 'handle region peptide' (HRP), mimics the handle region and thus will bind competitively to the receptor, thereby preventing receptor-mediated prorenin activation and reducing tissue RAS activity. Since prorenin is highly species-specific, different HRPs exist for humans, rats and mice.

HRP also binds with nanomolar affinity to the (P)RR (K_D ≈ 2-15 nM) (94, 95). *In vivo* studies in rats and mice support angiotensin-suppressing effects of HRP, as well as effects that were not related to angiotensin suppression (74, 76, 98). HRP doses ranged from 0.1 mg/kg per 28 days to 1 mg/kg per day. Campbell argued that Ang II, when administered under similar conditions at 0.3 mg/kg per day yields plasma levels of ≈150 pM (99). Since the decapeptide HRP is likely to be metabolized at least as rapidly as the octapeptide Ang II, the above infusion rates would yield HRP concentrations between 2 and 450 pM. Yet, Wilkinson-Berka *et al.*, when infusing HRP at 1 mg/kg per day in rats, were unable to detect intact HRP in blood plasma (100), and thus concluded that its levels were below the detection limit of their assay (3 pM). In contrast, Satofuka *et al.*, when administering HRP

intraperitoneally in mice, obtained peak HRP levels of ≈100 nM (98). The latter measurement did not include high-performance liquid chromatography to separate intact HRP from any crossreacting substance, and thus one explanation for this discrepancy might be that Satofuka's assay detected not only HRP, but also its metabolites and/or crossreacting substances. For instance, this assay recognized a baseline signal arising from endogenous prorenin. Clearly, more detailed information on the kinetics of HRP, obtained with appropriate assays and including tissue levels, is required to solve this issue.

In vitro studies investigating the blocking effect of HRP towards renin/prorenin binding or renin/prorenin-induced signaling are limited. Nurun Nabi *et al.* immobilized the human (P)RR on a sensor chip, making use of anti-(P)RR antibodies (95). Under these circumstances, 80 nM human HRP blocked the binding of both human prorenin and renin (applied at a concentration of 0.5 nM) by ≈30% (95). The blockade of renin binding is surprising, and the authors argued that HRP induces a conformational change in the (P)RR, thereby no longer allowing renin binding. Batenburg *et al.*, making use of rat VSMCs overexpressing the human (P)RR, observed no blocking effects of either human or rat HRP, even when using a concentration of 1 μM, towards human renin or human prorenin (both applied at a concentration of 20 nM) (12) The binding of ^{125}I-labeled human renin (0.1 nM) and human prorenin (0.2 nM) to U937 monocytes was also unaffected by 10 μM HRP (19). Moreover, although Feldt *et al.* observed increased Erk1/2 phosphorylation in U937 monocytes following exposure to 10 nM human renin or 2 nM human prorenin (19), 10 μM human HRP did not block this phenomenon. In contrast, mouse HRP blocked the mouse prorenin (2 nM)-induced Erk1/2 phosphorylation in mouse endothelial cells at concentrations of 10 and 100 μM (98). To explain these discrepancies, it has been proposed that, in certain cells, and possibly particularly *in vitro*, the (P)RR is not or scarcely located on the cell surface, thus not allowing an easy access for HRP (98). However, this leaves unexplained why renin/prorenin did have access to the (P)RR under these conditions. It is clear that the HRP concentrations (80 nM-100 μM) in the *in vitro* studies that did show inhibitory effects of this peptide (95, 98) were up to several orders of magnitude above the plasma levels of intact HRP *in vivo* (<3 pmol/L).

Consequently, an alternative explanation for the *in vivo* efficacy of HRP needs to be considered. Given the HRP-induced increases in phosphorylated Erk1/2 observed by some investigators, thus causing neuronal and glia injury in the retina, one possibility is that HRP acts as a partial agonist (100, 101). Although this could explain some of the contrasting effects towards renin/prorenin-induced signaling, it cannot explain the lack of effect of HRP on renin/prorenin binding. Thus, HRP might even exert effects that are not related to the (P)RR at all. Leckie and Bottrill were unable to demonstrate a specific binding site for HRP on human endothelial cells (102). Feldt *et al.* observed that fluorescein isothiocyanate-labeled HRP (FITC-HRP, 10 nM) bound to cells which expressed a (P)RR variant lacking the transmembrane region, so that binding should actually not have occurred (19). Fluorescence-activated cell sorter analysis did not reveal FITC-HRP (1 μM) binding in VSMCs expressing the human (P)RR without prior permeabilisation (with saponin) (103). Such binding did occur after permeabilisation, and was identical to that in wildtype cells not expressing the human (P)RR. Importantly, neither 20 nM human prorenin, nor 1 μM HRP affected FITC-HRP binding to VSMCs. These data indicate that HRP binds to an intracellular protein which is unlikely to be the (P)RR. In agreement with this conclusion, the nonspecific binding site observed by Leckie and Bottrill was located on cytoskeletal proteins (102). Recently, Nurun Nabi *et al.* demonstrated that the K_D of FITC-HRP is several orders of magnitude above that of HRP (96), so that any binding study involving FITC-HRP is unlikely to involve the (P)RR. Nevertheless, HRP did selectively block FITC-HRP binding, suggesting that both peptides do compete for the same binding site (19, 104). At this stage, thorough binding and competition assays are warranted, making use of labeled and unlabeled antagonists to clarify these discrepancies.

6. CONCLUSIONS

The concept of (pro)renin acting as a (P)RR agonist, inducing signaling independent from angiotensin generation, remains to be proven under *in vivo* circumstances. Data in transgenic animals with excessive prorenin levels do not support prorenin effects beyond Ang II generation, and the phenotype of (P)RR transgenic animals does not involve alterations in the RAS. Although *in vitro* studies do support (pro)renin effects that are mediated via the (P)RR, it should be taken into account that virtually all of these studies applied nanomolar (pro)renin that have no physiological relevance (11, 15-17, 19, 21-24, 26-28). Moreover, the prorenin that was used in these studies has not been characterized extensively. In addition, the (P)RR has now been identified as a component of the Wnt/β-catenin signaling pathway, being responsible for the interaction with the V-ATPase complex, even in the absence of (pro)renin. It is therefore clear that the (P)RR exerts important effects totally independent from the RAS. Indeed, (P)RR knockout, as opposed to RAS component knockout, is lethal, even when limited to cardiomyocytes. From this point of view the data obtained with the putative (P)RR antagonist, HRP, are confusing, even more while such data have been obtained by applying doses over a range of more than 100-fold, which yielded plasma concentrations ranging from < 3 pM to 100 nM (98, 100). Thus, at this stage, we need evidence that prorenin truly interacts with the (P)RR *in vivo* and that HRP blocks such interaction. An ideal model in this regard would be double transgenic rodents overexpressing the (P)RR and an active site-mutated prorenin that cannot display enzymatic activity. The tissue distribution of HRP should be investigated in great detail, also taking into consideration the predominant intracellular location of the (P)RR. Finally, the precise role of prorenin in facilitating (P)RR-V-ATPase interaction, if any, needs to be unraveled, e.g. making use of MDCK epithelial cells where the (P)RR is mainly present at the plasma membrane.

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