The (Pro)renin Receptor: Moving away from Prorenin?

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The (Pro)renin Receptor: Moving away from Prorenin?

De (Pro)renine Receptor: Is er nog een Rol voor Prorenine?

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Let life be beautiful like summer flowers and death like autumn leaves.

----Rabindranath Tagore

To my wife and my parents.

Table of Contents

Chapter 1	Introduction and Aim	9
Chapter 2	Renin- and Prorenin-Induced Effects in Rat Vascular Smooth Muscle Cells Overexpressing the Human (Pro)Renin Receptor: Does (Pro)Renin-(Pro)Renin Receptor Interaction Actually Occur?	27
Chapter 3	Urinary Renin and Angiotensinogen in Type 2 Diabetes: Added Value beyond Urinary Albumin?	43
Chapter 4	(Pro)renin Receptor Is Required for Prorenin-Dependent and -Independent Regulation of Vacuolar H^+-ATPase Activity in MDCK.C11 Collecting Duct Cells	57
Chapter 5	Tandem-affinity Purification of the (Pro)renin Receptor Reveals a Functional Interaction with Sortilin-1	73
Chapter 6	New Renin Inhibitor VTP-27999 Alters Renin Immunoreactivity and Does Not Unfold Prorenin	93
Chapter 7	Renin inhibitor VTP-27999 Differs from Aliskiren: Focus on Their Intracellular Accumulation and the (Pro)renin Receptor	115
Chapter 8	Summary and General Discussion	129
Samenvatting		141
Acknowledgement		149
Curriculum Vitae		153
Publication list		155
PhD Portfolio		157

Chapter 1

Introduction and Aim

HRP and Prorenin: Focus on the (Pro)renin Receptor and Vacuolar H^+-ATPase

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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 an accessory protein 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.

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 cicrculation. 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 been 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 an accessory protein

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.

(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)]. (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-β1 (TGF-β1), 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 V1 carrying out ATP hydrolysis and VO 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 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 (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 the emerging paradigm of protons as mediators of signal transduction pathways (58).

Since (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 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 VO domain of the V-ATPase, as knocking down the (P)RR decreases protein levels of VO, but not V1 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 by 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 AT1 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 cycle 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.

(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 C activation of MAP kinases and nuclear factors NF-κB and AP-1 (70), and induces expression of the proinflammatory factors interleukine-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-β1 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

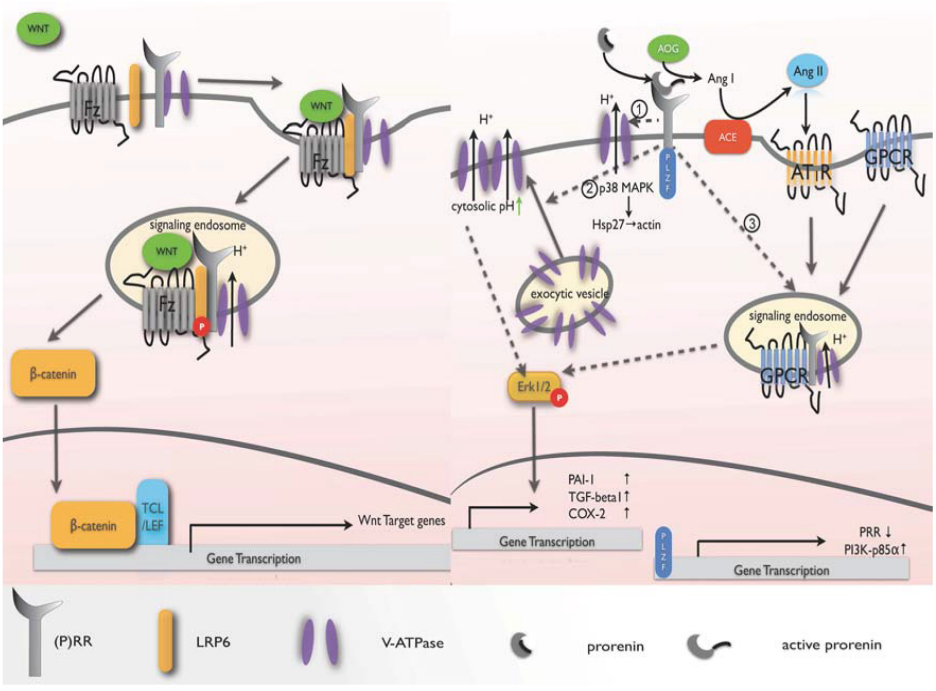

Figure 1. ***Schematic representation of the (P)RR and the V-ATPase in signal transduction pathways.*** Left panel: (P)RR in canonical Wnt signaling. Right panel: putative mechanisms of (P)RR in prorenin initiated signal transduction. See text for details. Abbreviations used: Ang, angiotensin; Aog, angiotensinogen; ACE, angiotensin-converting enzyme; AT1R, Ang II type 1 receptor; COX-2, cyclooxygenase-2; Erk, 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-β1, transforming growth factor-β1.

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 (AT1a) (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 that observed in diabetic patients, where increases are <5-fold (3) Plasma prorenin concentrations 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 that just prorenin levels alone.

Other unresolved issues are the localization and ligand affinity of (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).

HRP: a (P)RR antagonist?

As discussed, renin and prorenin bind with nanomolar affinity to the (P)RR, KD'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 KD 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 (KD ≈ 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 125I-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 mol/L 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 KD 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.

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, the highest of 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.

Aim of the thesis

The renin-angiotensin-system (RAS) plays a central role in regulating blood pressure and fluid balance. Overactivation of the RAS may increase blood pressure, and is thus one of the main causes for hypertension. Renin cleaves angiotensin I from angiotensinogen, and angiotensin I is converted by ACE to angiotensin II, the effector of the system that increases blood pressure. Independently of angiotensin generation, (pro)renin can directly activate intracellular signaling pathways via the (P)RR. This highlights a functional role of prorenin, which was thought to be only the inactive precursor of renin, despite the fact that its plasma level is 10-fold higher than that of renin.

(Pro)renin-(P)RR interaction is believed to affect the progression of cardiovascular disease based on in vivo and in vitro models. Yet, whether such interaction truly occurs under normal (patho)physiologic conditions is still a matter of debate, since supraphysiological (pro)renin levels are often required to observe effects. In addition, (pro)renin-independent (P)RR functions have been revealed over the past years. Thus, in this thesis, we aimed to study (pro)renin-(P)RR interaction, its signaling, and novel functions of the (P)RR.

In Chapter 2, we investigated (pro)renin-(P)RR interaction in vitro, and found that direct activation of (P)RR signaling by (pro)renin is unlikely to occur in vivo. At most, such interaction may occur in renin-synthesizing tissues, e.g. under pathologic conditions. In diabetes, prorenin is elevated and possibly originates in the collecting duct, where V-ATPases are highly expressed. In Chapter 3, we studied whether urinary renin and angiotensinogen can be used as a marker for renal RAS blockade during DRI treatment, AT1R blockade or both. Considering the high expression of the (P)RR in the collecting duct, and the fact that the (P)RR is an accessory protein of V-ATPase, we therefore studied the role of (pro)renin-(P)RR interaction in V-ATPase regulation in Chapter 4. Unexpectedly, we found that the (P)RR is required for both (pro)renin–dependent and –independent regulation of V-ATPase. To better understand the (P)RR and to discover novel functions of this receptor, in Chapter 5, we identified (P)RR-interacting proteins using a tandem affinity purification method. Our results revealed potentially important roles of the (P)RR in signaling and lipid metabolism.

Blockade of the RAS is a main way to control blood pressure in clinical practice. Unfortunately, when using an ACE inhibitor or an AT1R blocker, often increases in plasma renin activity (PRA) are observed, which may result in a so-called angiotensin II escape. No such rises in PRA should occur during renin inhibition, raising the possibility that direct renin inhibitors (DRI) are more effective in lowering blood pressure than other RAS blockers. In Chapters 6 & 7, we compared two DRIs, aliskiren and VTP-27999, focusing on their intracellular accumulation and their interference with (pro)renin-(P)RR signaling. Chapter 8 puts all data into perspective and provides directions for future studies.

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Chapter 2

Renin- and Prorenin-Induced Effects in Rat Vascular Smooth Muscle Cells Overexpressing the Human (Pro)Renin Receptor

Does (Pro)Renin-(Pro)Renin Receptor Interaction Actually Occur?

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Abstract

Renin/prorenin binding to the (pro)renin receptor ([P]RR) results in direct (angiotensin-independent) second-messenger activation *in vitro*, whereas *in vivo* studies in rodents overexpressing prorenin (≈400-fold) or the (P)RR do not support such activation. To solve this discrepancy, DNA synthesis, extracellular signal–regulated kinase 1/2 phosphorylation, and plasminogen-activator inhibitor 1 release were evaluated in wild-type and human (P)RR-overexpressing vascular smooth muscle cells after their incubation with 1 to 80 nmol/L of (pro)renin. Human prorenin (4 nmol/L, ie, ≈800-fold above normal) + angiotensinogen increased DNA synthesis in human (P)RR cells only in an angiotensin II type 1 receptor–dependent manner. Prorenin at this concentration also increased plasminogen-activator inhibitor 1 release via angiotensin. Prorenin alone at 4 nmol/L was without effect, but at 20 nmol/L (≈4000-fold above normal) it activated extracellular signal–regulated kinase 1/2 directly (ie, independent of angiotensin). Renin at concentrations of 1 nmol/L (≈2000-fold above normal) and higher directly stimulated DNA synthesis, extracellular signal–regulated kinase 1/2 phosphorylation, and plasminogen-activator inhibitor 1 release in wild-type and human (P)RR cells, and similar effects were seen for rat renin, indicating that they were mediated via the rat (P)RR. In conclusion, angiotensin generation depending on prorenin-(P)RR interaction may occur in transgenic rodents overexpressing prorenin several 100-fold. Direct (pro)renin-induced effects via the (P)RR require agonist concentrations that are far above the levels in wild-type and transgenic rats. Therefore, only prorenin (and not [P]RR) overexpression will result in an angiotensin-dependent phenotype, and direct renin-(P)RR interaction is unlikely to ever occur in nonrenin-synthesizing organs.

Introduction

Since the discovery of the (pro)renin receptor ([P]RR) [1], many investigators have attempted to unravel its relationship with the renin-angiotensin (Ang) system. Its overexpression resulted in elevated blood pressure, a rise in plasma aldosterone levels, increased renal cyclooxygenase 2 expression, and glomerulosclerosis, albeit in the absence of changes in renin-Ang system component levels [2,3]. Vice versa, prorenin overexpression, elevating plasma prorenin levels ≤400-fold, did raise blood pressure in an Ang-dependent manner. Yet, it did not result in fibrosis and/or glomerulosclerosis [4–6], although this was expected on the basis of in vitro studies showing that direct prorenin-(P)RR interaction (ie, independent of Ang) results in activation of the extracellular signal–regulated kinase (ERK) 1/2 pathway, thereby upregulating profibrotic genes like transforming growth factor-β1 (TGF-β1) and plasminogen-activator inhibitor 1 (PAI-1) and increasing cell proliferation [7–11].

The (P)RR colocalizes with vacuolar H^+-ATPase (V-ATPase) in the kidney [12]. This may relate to the observation that the 8.9-kDa accessory protein ATP6AP2 of vacuolar H^+-ATPase is a posttranslationally truncated version of the (P)RR, resembling its C-

terminal domain. V-ATPases play an important role in the acidification of subcellular compartments. The (P)RR is indispensable for V-ATPase integrity, as in cardiomyocyte-specific (P)RR knockout mice, the abundance of several V-ATPase subunits is decreased in the cardiomyocytes, resulting in the development of heart failure because of defective autophagy and, ultimately, cell death.13 Moreover, the (P)RR functions as an adaptor between V-ATPase and receptors for members of the Wnt family of signaling molecules [14]. These findings clearly indicate the importance of the (P)RR beyond prorenin binding.

Both renin and prorenin are believed to bind to the (P)RR, with dissociation constants ranging from ≈1 to 20 nmol/L depending on the use of immobilized receptors or membrane fractions of (P)RR-transfected cells [1,15,16]. When comparing renin and prorenin in the same assay, most studies revealed that the dissociation constant of prorenin for the human (P)RR is 3- to 4-fold lower than that of renin. Nanomolar dissociation constants are difficult to reconcile with the picomolar levels of renin (≈0.5 pmol/L) and prorenin (≈5.0 pmol/L) in extracellular fluid [17], but explain why, in most in vitro studies, investigating (pro)renin-(P)RR interaction high nanomolar (≤100 nmol/L) (pro)renin concentrations were required to observe effects [10]. A peptidic antagonist has been designed based on the idea that the prosegment of prorenin contains a "handle region" (10P to 19P) that binds to the receptor, allowing prorenin to become catalytically active [18]. This handle region peptide (HRP) mimics the handle region and, thus, should bind competitively to the receptor, thereby preventing receptor-mediated prorenin activation and reducing tissue renin-Ang system activity. Because prorenin is highly species specific, different HRPs exist for humans, rats, and mice. Although beneficial effects of these HRPs have been obtained in animal models [19,20], clear evidence that this peptide blocks prorenin-(P)RR interaction is lacking. It has even been claimed that HRP also prevents renin binding to the (P) RR, acts as an agonist of the (P)RR, and/or has (P)RR-independent effects [16,21,22].

To obtain a better understanding of the apparent discrepancy between the in vitro and in vivo consequences of (pro)renin-(P)RR interaction, we carefully compared the concentration dependency of the effects of human renin and prorenin via the human (P)RR (h[P]RR) in aortic vascular smooth muscle cells (VSMCs) obtained from rats overexpressing the h(P)RR in VSMCs and their wild-type controls [3]. We focused on Ang II formation and signaling, making use of putative blockers of the (P)RR (human and rat HRP) and V-ATPase (bafilomycin), in addition to knocking down the (P)RR with small interfering (si) RNA. Rat VSMCs have the advantage that they allow us to simultaneously study effects of human renin/prorenin that are mediated via the rat (P)RR and/or mannose 6-phosphate receptors (M6PRs). The latter receptors are clearance receptors for both renin and prorenin, but have also been linked to signaling [23,24]. Both receptor subtypes might mediate effects of (human) renin/prorenin when overexpressed in rats or mice.

Methods

Cell Culture and Transfection

All of the experiments were performed according to the regulations of the Animal Care Committee of the Erasmus MC, in accordance with the Guiding Principles of the American Physiological Society. Primary cultures of VSMCs were prepared from aortas of 6-week–old transgenic rats with VMSC expression of the h(P)RR3 and their control littermates (n=8 for both) [25]. In short, VSMCs were isolated from the aorta, pooled, plated, and maintained at 37°C in a humidified 5% CO2 incubator in supplemented SmBM-2 medium (Cambrex) containing 10% FBS. Cells were cultured to confluence in a 75-cm^2 flask (in supplemented SmBm-2 medium; passages 3–8), trypsinized, and seeded into 6- or 24-well plates using the above medium. This yielded a confluent monolayer of $\approx 4\times10^5$ cells per centimeter squared after 3 days. When indicated, cells were transfected with a mixture of siRNA against the rat and human (P)RR or control siRNA (rat [P]RR siRNA: 5'-GAGAAUGCAGUUCCCUU-3', 5'-CAACCUUGCGUAUAAGUAU-3',5'-CAACAUUUGGAAUAUGGAU-3'; human [P]RR siRNA:5'-GGACUAUCCUUGAGGCAAA-3', 5'-GGUCUGUUGUUUUCCG-AAA-3', 5'-GAGUGUAUAUGGUAGGGAA-3'; control siRNA: silencer negative control [Ambion, Applied Biosystems]) by nucleofection following the recommendations of the manufacturer (Amaxa, Gaithersburg, MD). Briefly, 10^6 cells in suspension were incubated with 1 μmol/L of siRNA. Cells were allowed to recover overnight with 10% FCS. Before the start of an experiment, the cells were cultured for 24 hours under serum-free conditions.

Prorenin and Renin Preparations

Recombinant human prorenin was a kind gift of Dr. W. Fischli (Actelion, Basel, Switzerland; stock concentration 0.8 mg/mL). Recombinant rat prorenin was a kind gift of Dr D. Day (Molecular Innovations, Novi, MI; stock concentration 0.4 mg/mL). Both were converted to renin with trypsin as described before [26].

Binding Studies With Recombinant Rat and Human Renin and Prorenin

To study the consequence of transfection on the binding of human prorenin, as well as to quantify the binding of rat renin and prorenin, taking into consideration the contribution of both M6PRs and (P)RRs to this process, cells (before or after transfection) were incubated at 37°C with 20 to 80 nmol/L of recombinant rat or human renin/prorenin for 4 hours in the presence or absence of 10 mmol/L of M6P (to block M6PRs). At the end of the incubation period, the culture medium was removed, and the cells were washed 3 times with 1 mL of ice-cold PBS before they were lysed with ice-cold 0.2% triton X-100 in PBS. Cell lysates were quickly frozen in liquid N2 and stored at −70°C. Rat renin and prorenin in the cell lysates (the latter after its conversion to renin by incubation of the sample for 48 hours at 4°C with 0.5 caseinolytic units per milliliter of plasmin) were measured by enzyme-kinetic assay in the presence of excess sheep substrate [27]. Human renin and prorenin (the latter after a 48-hour incubation at 4°C with 10 μmol/L of aliskiren to allow its recognition by an active site-directed

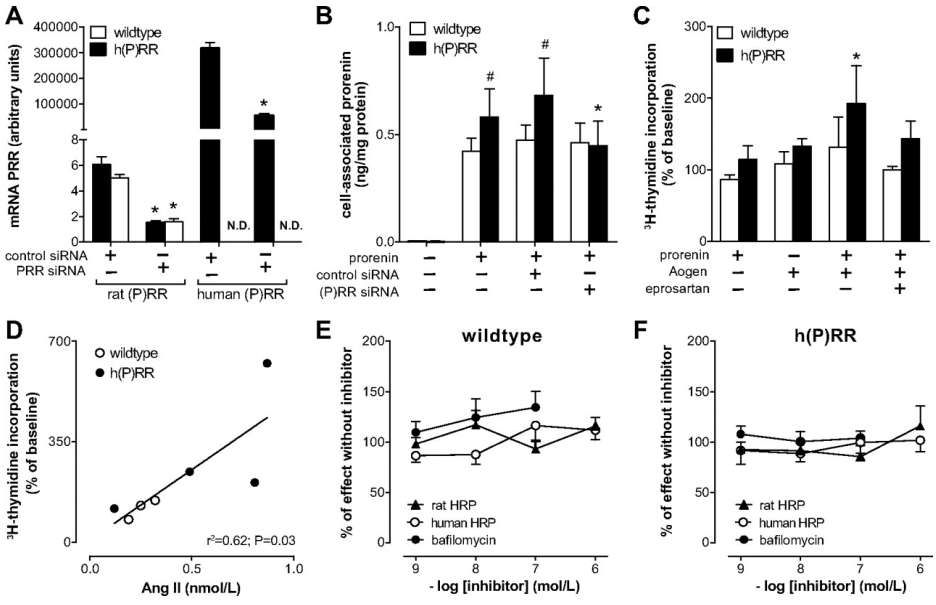

Figure 1. A, Rat and human (pro)renin receptor ([P]RR) expression in wild-type and human (h) (P)RR vascular smooth muscle cells (VSMCs) before and after transfection with (P)RR or mock (negative control) small interfering RNA (siRNA; mean±SEM of n=6). B, Binding of human prorenin to wild-type and h(P)RR VSMCs before and after transfection with (P)RR or mock siRNA (mean±SEM of n=15). C, 3H-thymidine incorporation in wild-type and h(P)RR VSMCs after incubation with 4 nmol/L of prorenin ± 150 nmol/L of angiotensinogen (Aogen) with or without 1 µmol/L of eprosartan (mean±SEM of n=7). D, Relationship between the medium Ang II levels and the increase in 3H-thymidine incorporation. E and F, Effect of rat handle region peptide (HRP), human HRP, or bafilomycin on the effect of 4 nmol/L of prorenin +150 nmol/L angiotensinogen on 3H-thymidine incorporation in wild-type (E) and h(P)RR (F) cells (mean±SEM of n=6–8). *P<0.05 vs control, #P<0.05 vs wild-type.

antibody) [28] were measured by a renin-specific immunoradiometric assay (Cisbio).

DNA Synthesis

To study the effect of human renin and prorenin on DNA synthesis, cells were incubated at 37°C with 1 to 4 nmol/L of recombinant human renin or prorenin for 24 hours in the absence or presence of 150 nmol/L of human angiotensinogen (Sigma), 10 mmol/L of M6P, 1 µmol/L of eprosartan (Ang II type 1 receptor antagonist), 1 µmol/L of PD123319 (Ang II type 2 receptor antagonist), 10 µmol/L of aliskiren, 1 nmol/L to 1 µmol/L of rat HRP (RILLKKMPSV-OH, to block the rat [P]RR), 1 nmol/L to 1 µmol/L of human HRP (IFLKRMPSI-OH, to block the human [P]RR; Biosyntan, Berlin, Germany), or 1.0 nmol/L to 0.1 µmol/L of bafilomycin (inhibitor of V-ATPase). As a positive control, 100 nmol/L of Ang II was used. The last 6 hours of the incubation period, 3H-thymidine was added to a final concentration of 0.5 µCi/mL in each well. 3H-Thymidine incorporation was measured with a scintillation counter.

(P)RR Expression and ERK1/2 Phosphorylation (Pro)Renin Receptor

To verify whether transfection had been successful, transfected and nontransfected cells were lysed using Nonidet P-40 lysis buffer and kept on ice for ≥15 minutes. Next, the cell lysates were centrifuged at 14,000x g for 15 minutes at 4°C. Supernatants were collected and stored at −20°C. Western blotting was performed with 10 μg of protein using an antibody that recognizes both the human and rat (P) RR (anti-ATP6AP2, 1:1000, Sigma). A peroxidase-conjugated antibody (goat antirabbit, 1:5000) was used to visualize the receptor. Total mRNA was isolated from TRIzol-lysed cells with a combined protocol of QIAzol lysis reagent and Qiagen RNeasy minikit (including the RNase-Free DNAse set; Qiagen) according to the manufacturer's protocol. RNA was reverse transcribed into cDNA by using the Transcriptor First-Strand cDNA synthesis kit from Roche Diagnostics. Expression was measured by real-time quantitative PCR on an ABI 7500 Fast Sequence Detection System (PE Biosystems). Primer and probes (Biotez) were designed with PrimerExpress 3.0 (Applied Biosystems). Eukaryotic 18S rRNA was used as endogenous control. The following primers were used: rat (P)RR: forward (5'-CCCCACAGGCAGCGTC-3'), reverse (5'-GAATTGGCCACACTGTCAAGAC-3'), probe: (5'-FAM-CTCTTACCCTTGGAGAATGCAGTTCCCTT-TAMRA-3'); human (P)RR: forward (5'-CCCAGACGTGGCTGCATT-3'), reverse (5'-GCGAGTCCTGGCCAAGAA-3'), probe (5'-FAM-TCCATGGGCTTCTCTGTGAAAGAAGACC-TAMRA-3'); and 18S: forward (5'-ACATCCAAGGAAGGCAGCAG-3'), reverse (5'-TTTTCGTCACTACCTCCCCG-3'),probe(5'-FAM-CGCGCAAATTACCCACTC-CCGACTAMRA-3').

Extracellular Signal–Regulated Kinase 1/2

To study the effects of renin and prorenin on ERK1/2 phosphorylation, cells were incubated at 37°C with 4 to 80 nmol/L of recombinant human or rat (pro)renin for maximally 60 minutes in the absence or presence of 1 μmol/L of eprosartan, 1 μmol/L of PD123319, and/or 150 nmol/L of angiotensinogen. As a positive control, 100 nmol/L of Ang II was used. Cells were processed as described above. Western blotting was performed with 10 μg of protein using antibodies (Cell Signaling) for phosphorylated p42/44 mitogen-activated protein kinase and total p42/44 mitogen-activated protein kinase (phosphorylated ERK1/2 and ERK1/2, respectively, diluted 1:1000). A peroxidase-conjugated antibody (goat antirabbit, 1:5000) was used to visualize phosphorylated ERK1/2 and ERK1/2. Results were expressed as percentage of phosphorylated p42/44 mitogen-activated protein kinase of total p42/44 mitogen-activated protein kinase.

TGF-β1, PAI-1, Total Protein, and DNA

To study the effects of renin and prorenin on TGF-β1 and PAI-I, cells were incubated at 37°C with buffer (control), 4 nmol/L of recombinant human (pro)renin (with or without 150 nmol/L of human angiotensinogen), or 0.3 to 15.0 nmol/L of recombinant rat (pro)renin in the presence or absence of the ERK1/2 inhibitor PD98059 (10 μmol/L) for maximally 24 hours. FCS (5%) and Ang II (100 nmol/L) were used as positive controls. The medium was harvested and the cells were lysed in 0.2% Triton

X-100. The samples were frozen at −20°C. Rat TGF-β1 levels were determined using the Quantikine TGF-β1 ELISA kit (R&D Systems). Rat PAI-I levels were measured using the Zymutest ELISA kit (Hyphen Biomed). Total protein was determined according to Bradford, and total DNA was quantified with 4',6-diamidino-2-phenylindole.

Data Analysis

Results are expressed as mean±SEM. Each experiment was performed in triplicate, and the n number refers to the number of triplicate experiments. Statistical analysis was performed using a t test or 1-way or 2-way ANOVA where appropriate. P<0.05 was considered significant.

Results

Transfection and Recombinant Human Prorenin Binding

Transfection with (P)RR siRNA, but not mock siRNA, greatly suppressed (P)RR (human and/or rat) expression in wild-type and h(P)RR cells (Figure 1A; n=6), and under these conditions, Western blot analysis no longer allowed the detection of the (P)RR in either cell type (data not shown; n=6). In parallel, the enhanced prorenin binding in h(P)RR cells (P<0.05 versus wild-type) decreased to wild-type levels after siRNA transfection (Figure 1B; n=15), suggesting that this enhanced binding exclusively represented binding to h(P)RRs.

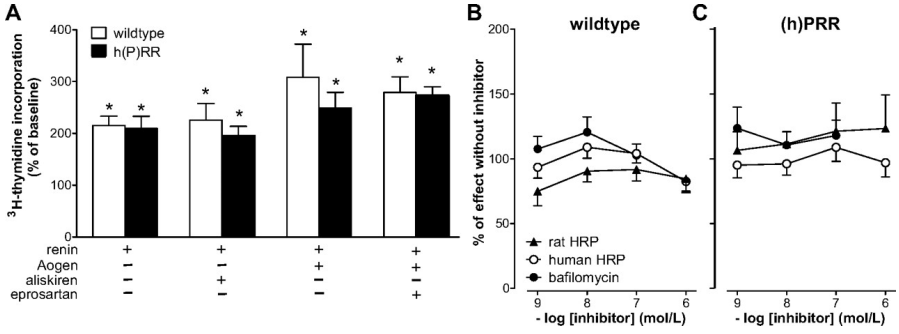

Figure 2. A, 3H-thymidine incorporation in wild-type and human (h) (pro)renin receptor ([P]RR) vascular smooth muscle cells (VSMCs) after incubation with 1 nmol/L of renin (R) ± 150 nmol/L of angiotensinogen (Aogen) in the absence or presence of 10 μmol/L of aliskiren or 1 μmol/L of eprosartan (mean±SEM of n=12). B and C, Effect of rat handle region peptide (HRP), human HRP, or bafilomycin on the effect of 1 nmol/L of renin + 150 nmol/L of angiotensinogen on 3H-thymidine incorporation in wild-type (B) and h(P)RR (C) cells (mean±SEM of n=6–8). *P<0.05 vs baseline.

DNA Synthesis Induced by Recombinant Human Renin and Prorenin

Prorenin (4 nmol/L) alone (n=7) and angiotensinogen alone (n=7) were without effect on DNA synthesis in both wild-type and h(P)RR cells (Figure 1C). However, in combination, they almost doubled DNA synthesis in h(P)RR cells (n=7; P<0.05), without being effective in wild-type cells (n=7). Ang II increased DNA synthesis to the same degree (169±23% versus 146±22%) in both cell types (n=19). Eprosartan (n=7) but not PD123319 (data not shown; n=7) prevented the combined prorenin/angiotensinogen

effects in h(P)RR cells, indicating that they depended on Ang II generation and subsequent Ang II type 1 receptor activation. Indeed, the Ang II level in the medium of these cells after the addition of prorenin + angiotensinogen correlated directly with the degree of 3H-thymidine incorporation (Figure 1D). The lack of effect of prorenin alone is in agreement with the fact that these VSMCs do not synthesize angiotensinogen themselves [15,28]. Rat HRP (n=6), human HRP (n=8) and bafilomycin (n=6) did not affect the DNA synthesis induced by prorenin + angiotensinogen (Figure 1E and 1F). Renin at 1 nmol/L doubled DNA synthesis in both cell types (n=6; P<0.05), and this effect was not altered by the addition of angiotensinogen, aliskiren, or eprosartan (Figure 2A; P value not significant between treatments). This indicates that renin does not exert its effects on DNA synthesis via Ang II or the h(P)RR. M6P did not affect DNA synthesis in either cell type (data not shown; n=7), suggesting that the effect of renin also did not involve M6PR. Rat HRP (n=6), human HRP (n=8), and bafilomycin (n=6) did not affect the DNA synthesis induced by renin + angiotensinogen (Figure 2B and 2C).

ERK1/2 Phosphorylation Induced by Recombinant Human Renin and Prorenin

Angiotensin II identically increased ERK1/2 phosphorylation in wild-type (n=8) and h(P)RR cells (n=8), a maximum being reached after ≈15 minutes (P<0.01 for both; Figure 3). Eprosartan and PD123319 did not affect ERK1/2 phosphorylation (n=6; data not shown). Prorenin at 4 nmol/L did not increase ERK1/2 phosphorylation in either cell type (n=8), but at 20 nmol/L, in the presence of eprosartan and PD123319, it doubled ERK1/2 phosphorylation (to 233±49% of baseline) after 5 minutes exclusively in h(P)RR cells (Figure 4A; P<0.05). This suggests that the effect occurred via h(P)RR activation. Indeed, (P)RR siRNA (but not mock siRNA) transfection fully prevented this effect (n=6; Figure 4A). Yet, 1 µmol/L of human HRP did not block it (199±16%; n=6), nor did 10 µmol/L of aliskiren (224±100%; n=3). Prorenin (4 nmol/L) + angiotensinogen tended to increase ERK1/2 phosphorylation (P=0.07) in h(P)RR cells (by 40±14% after 15 minutes; n=3) but not (P value not significant) in wild-type cells (increase of 33±27%; n=3).

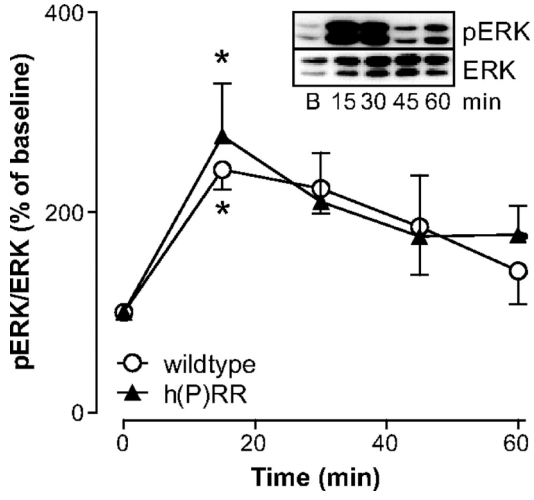

Figure 3. Extracellular signal–regulated kinase (ERK) 1/2 phosphorylation (ratio of phosphorylated ERK1/2 versus total ERK1/2) in wild-type and human (h) (pro) renin receptor ([P]RR) vascular smooth muscle cells (VSMCs) after incubation with 100 nmol/L of angiotensin II for maximally 60 minutes (mean±SEM of n=8). Insert, representative blot (B indicates baseline). *P<0.05 vs baseline.

Renin at 4 nmol/L did not affect ERK1/2 phosphorylation in either cell type (n=8; Figure 4B), but at 20 nmol/L, in the presence of eprosartan and PD123319, it identically increased ERK1/2 phosphorylation in both cell types at 5 minutes (n=12). This effect was unaltered in the presence of 1 μmol/L of human HRP or 10 μmol/L aliskiren (n=6 for each; data not shown), suggesting that it involved neither the h(P)RR nor Ang II.

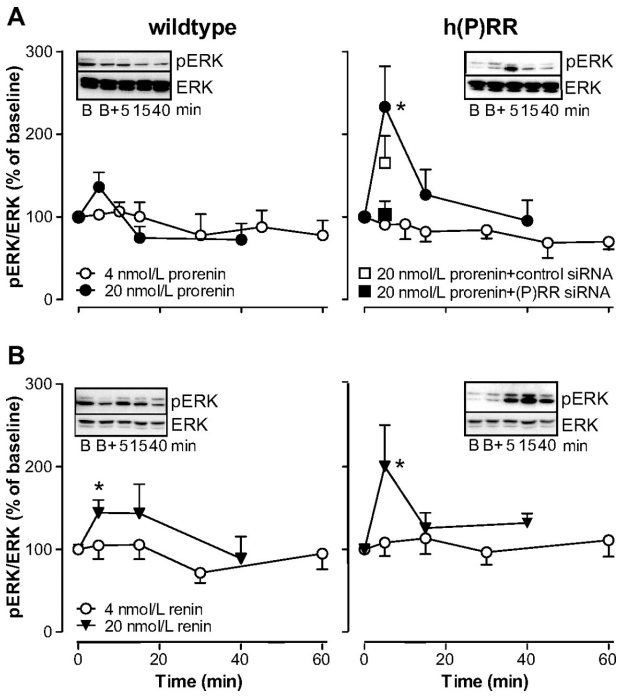

Figure 4. A and B, Extracellular signal–regulated kinase (ERK) 1/2 phosphorylation (ratio of phosphorylated ERK1/2 versus total ERK1/2) in wild-type and human (h) (pro)renin receptor ([P]RR) vascular smooth muscle cells (VSMCs) after incubation with 4 or 20 nmol/L of recombinant human prorenin (A) or renin (B) with 1 μmol/L of eprosartan and 1 μmol/L of PD123319, in the absence or presence of (P)RR or mock (negative control) small interfering RNA (siRNA), for maximally 60 minutes (mean±SEM of n=6). Insert, representative blot (B indicates baseline; B+, baseline+eprosartan+PD1233319). *P<0.05 vs baseline.

Binding of Recombinant Rat Renin and Prorenin and Their Effect on ERK1/2 Phosphorylation

Both cell types bound recombinant rat renin and prorenin to the same degree (n=3), and pretreatment with M6P suppressed this binding by ≈80% (Figure 5A). This indicates that both VSMC types bind rat renin and prorenin predominantly via M6PRs. Recombinant rat renin (but not prorenin) concentration-dependently increased ERK1/2 phosphorylation in both cell types (n=5), although significance was reached for renin at a concentration of 40 nmol/L only (Figure 5B). In the presence of M6P, the effect of 40 nmol/L renin in wild-type cells doubled from 211±53% to 395±106% (n=6; P<0.05). This suggests that rat renin mediates its effects on ERK1/2 phosphorylation via a non-M6PR receptor and that this effect is enhanced when M6PRs are occupied. Recombinant rat renin increased PAI-1 release from both cell types (n=6; Figure 5C), whereas recombinant rat prorenin (in the absence of angiotensinogen; n=6) was without effect.

TGF-β1 and PAI-1 Release Induced by Recombinant Human or Rat Renin and Prorenin

Neither recombinant human renin (4 nmol/L) nor recombinant human prorenin (4 nmol/L) affected TGF-β1 release from wild-type or h(P)RR cells (n=6–8; Figure 6A). They also did not affect the cellular TGF-β1 levels after 24 hours (data not shown). Ang II (100 nmol/L) also did not alter TGF-β1 release or the cellular TGF-β1 levels in both cell types (n=3). Yet, 5% FCS increased TGF-β1 release by 270±33% (n=3; P<0.05) after 24 hours.

Ang II (100 nmol/L) and recombinant human renin (4 nmol/L) increased PAI-1 release from both cell types to the same degree (n=6; P<0.01; Figure 6B). Coincubating 4 nmol/L of human renin with human angiotensinogen further increased its effect (n=6; Figure 6C; P<0.05), suggesting that the effects of renin and Ang II are additive. PD98059 (10 μmol/L) reduced the effect of renin by 18±6.4% and 21±5.9% in wild-type and h(P)RR cells, respectively (n=6; P<0.05). Prorenin (4 nmol/L) only increased PAI-1 release in the presence of angiotensinogen (Figure 6C; n=6), and its effect was most prominent in h(P)RR cells. Unfortunately, for unknown reasons, transfection with either mock siRNA or (P)RR siRNA doubled baseline PAI-1 release (n=6; data not shown), thereby not allowing a reliable estimation of the (pro)renin-induced effects on PAI-1 via the (P)RR.

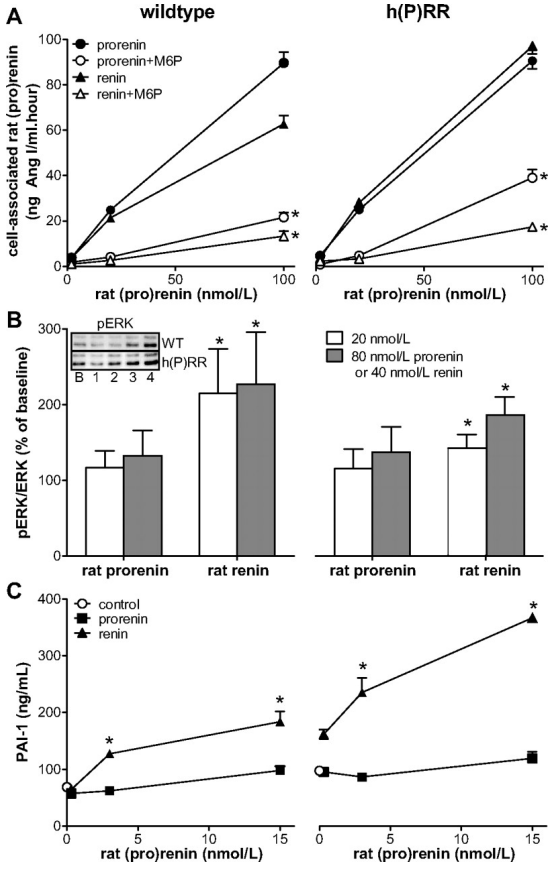

Figure 5. A, Binding of recombinant rat prorenin and renin to wild-type and human (h) (pro)renin receptor ([P]RR) vascular smooth muscle cells (VSMCs) in the absence or presence of 10 μmol/L of M6P (mean±SEM of n=3). B, Extracellular signal–regulated kinase (ERK) 1/2 phosphorylation (ratio of phosphorylated ERK1/2 versus total ERK1/2) in wild-type and h(P)RR VSMCs after a 5-minute incubation with 20 to 80 nmol/L of recombinant rat prorenin or renin in the presence of 1 μmol/L of eprosartan and 1 μmol/L of PD123319 (mean±SEM of n=5). Insert, Representative blot (B indicates baseline; 1=20 nmol/L prorenin; 2=80 nmol/L prorenin; 3=20 nmol/L renin; 4=40 nmol/L renin). C, Plasminogen-activator inhibitor 1 (PAI-1) release from wild-type and h(P)RR VSMCs after a 24-hour incubation with recombinant rat prorenin or renin (mean±SEM of n=6). *P<0.05 vs without M6P (A) or baseline (B and C).

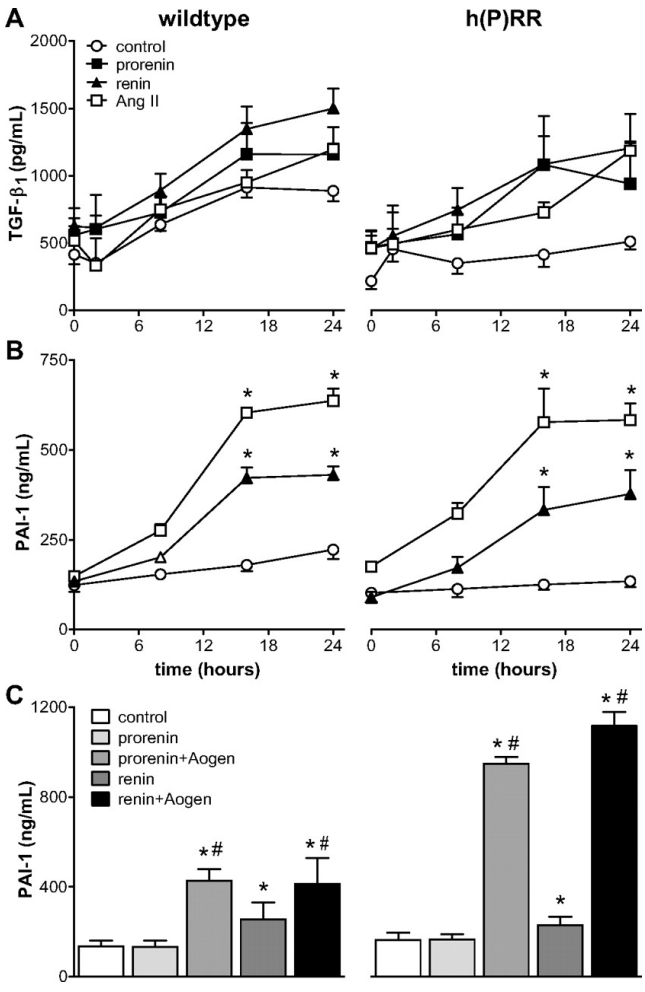

Figure 6. A, Transforming growth factor-β1 (TGF-β1) release from wild-type and human (h) (pro)renin receptor ([P]RR) vascular smooth muscle cells (VSMCs) after incubation with buffer, 100 nmol/L of angiotensin (Ang) II, 4 nmol/L of recombinant human prorenin, or 4 nmol/L of recombinant human renin (mean±SEM of n=6). B, Plasminogen-activator inhibitor 1 (PAI-1) release from wild-type and h(P)RR VSMCs after incubation with buffer, 100 nmol/L of Ang II, or 4 nmol/L of recombinant human renin (mean±SEM of n=6). C, Release of PAI-1 from wild-type and h(P) RR VSMCs after a 24-hour incubation with buffer, 4 nmol/L of renin, or 4 nmol/L of recombinant human prorenin ± 150 nmol/L of angiotensinogen (mean±SEM of n=6). *P<0.05 vs baseline. #P<0.05 vs (pro)renin alone.

Discussion

This study shows that low nanomolar (4 nmol/L) concentrations of human prorenin, in the presence of the h(P)RR, yield angiotensin levels that are sufficient to stimulate DNA synthesis via Ang II type 1 receptor activation. It must be realized that a prorenin concentration of 4 nmol/L is 2 to 3 orders of magnitude higher than the in vivo plasma concentration of prorenin [17,29]. Thus, such prorenin-(P)RR interaction is unlikely to be of physiological relevance in nonprorenin-synthesizing tissues like the heart and vascular wall, because in such organs the interstitial fluid levels of prorenin, at most, will resemble those in blood plasma [27]. Even higher (20 nmol/L) prorenin concentrations were required to directly (ie, independent of angiotensin) stimulate ERK1/2 phosphorylation via the h(P)RR. Angiotensin generation in these latter studies could be ruled out, because VSMCs do not synthesize angiotensinogen [15,28], angiotensinogen

had not been added to the medium, and the effect occurred despite the presence of Ang II type 1 and Ang II type 2 receptor antagonists. Similar data have been obtained in a variety of cell types, including endothelial cells, mesangial cells, and monocytes [7–9, 30], and the required prorenin concentrations were almost always (far) above 1 nmol/L. Interestingly, Liu *et al* recently claimed a direct effect of 20 nmol/L of rat prorenin on 3H-thymidine incorporation in rat VSMCs [11]. When using a cell proliferation assay (Promega), we were able to confirm that such prorenin concentrations, in h(P)RR VSMCs only, increased proliferation by 25±4.4% (n=6; data not shown). Thus, it appears that significant angiotensin generation resulting from prorenin-(P)RR interaction occurs at lower prorenin levels than direct ERK1/2 activation and DNA synthesis. This could explain why, in rodent models with maximally 400-fold elevated plasma prorenin levels, only Ang II–dependent effects were observed 4–6 and no direct prorenin effects.

In contrast to prorenin, human renin, at concentrations ranging from 1 to 20 nmol/L (i.e, ≈4–5 orders of magnitude above the normal renin levels in blood plasma) increased both DNA synthesis and ERK1/2 phosphorylation in an angiotensin and h(P)RR-independent manner. This conclusion is based on our observation that these effects were unaltered in the presence of angiotensinogen, aliskiren, and/or the h(P)RR. Because both rat (P)RR and M6PR occur in wild-type and h(P)RR VSMCs [15,28], human renin may have acted via one of these receptors. Indeed, rat renin (but not rat prorenin) also stimulated ERK1/2 phosphorylation. Moreover, both cell types bound rat renin and prorenin to the same degree, and this binding occurred for >80% via M6PRs. Yet, M6P, if anything, enhanced the effect of rat renin on ERK1/2 phosphorylation, demonstrating that this effect did not involve M6PR stimulation. Possibly M6PR blockade facilitated rat renin binding to rat (P)RRs, thus increasing its effect via these receptors. M6P did not affect DNA synthesis, implying that the effect of human renin on DNA synthesis also did not involve M6PRs. Taken together, the effects of human renin and rat renin on ERK1/2 and DNA synthesis are most likely mediated via rat (P)RRs. In agreement with this concept, binding of human (pro)renin to the rat (P)RR (and vice versa) has been demonstrated before [31]. Rat prorenin apparently is less potent than rat renin toward the rat (P)RR. This opposes the findings on the h(P)RR, for which prorenin appeared to be the endogenous agonist [15]. Nevertheless, transgenic rats overexpressing human renin (which does not react with rat angiotensinogen) are healthy and do not display hypertension or fibrosis [32]. The reason for this is that the plasma levels of human renin levels in such rats (226±58 pg/mL, n=6; M. Bader and A.H.J. Danser, unpublished observation), are at most ≈1 order of magnitude above the normal levels in humans, that is, far below the levels required to induce direct effect via the rat (P)RR.

In addition to DNA synthesis, direct renin/prorenin-induced ERK1/2 activation has been reported to result in the synthesis and release of both TGF-β1 and PAI-1. We were unable to show an effect on TGF-β1 when applying 4 nmol/L of human renin to VSMCs. This was not because of our inability to show an effect on TGF-β1, because 5% serum greatly increased the TGF-β1 production, in full agreement with previous data [8]. Human renin (4 nmol/L), like rat renin, did induce PAI-1 release, and ERK1/2

blockade with PD98059 partially blocked this effect. The effect occurred to the same degree in wild-type and h(P)RR VSMCs, ruling out a role for the h(P)RR. Unfortunately, deleting the (P)RR with (P)RR siRNA affected baseline PAI-1 release, thus not allowing us to establish firmly whether the effect on PAI-1 truly involved the rat (P)RR. Nevertheless, the inability of rat prorenin to exert the same effect mimicked our findings on ERK1/2 phosphorylation. Most likely, therefore, all of the effects of rat and human renin on DNA synthesis, ERK1/2 phosphorylation, and PAI-1 release involve the same phenomenon, that is, stimulation of the rat (P)RR. In an earlier study in cardiomyocytes, we observed that prorenin increased PAI-1 release only in the presence of angiotensinogen, that is, in an angiotensin-dependent manner, like its effect on DNA synthesis [23]. Indeed, Ang II greatly increased PAI-1 release from VSMCs, and prorenin (4 nmol/L) only stimulated PAI-1 release in the presence of angiotensinogen. Moreover, the effect of renin (4 nmol/L) combined with angiotensinogen was much bigger than that of renin alone, suggesting that the effects of renin (via the rat [P]RR) and Ang II are additive.

Although the siRNA data confirm the h(P)RR dependency of the enhanced prorenin binding and ERK1/2 activation in h(P)RR cells, both human HRP and rat HRP in our hands were without any effect toward either prorenin or renin. This has been noted before [7,15] and raises the possibility that the beneficial effects of these drugs involve other, as-yet-undefined targets [19-20,22]. Bafilomycin also did not affect the effect of renin and prorenin on DNA synthesis, suggesting that this phenomenon, unlike acidification [12], does not involve V-ATPase.

Perspectives

Prorenin-(P)RR interaction resulting in Ang II generation requires prorenin levels that are 2 to 3 orders of magnitude above its normal plasma levels. Signaling derived from direct (pro)renin-(P)RR interaction requires renin and prorenin levels that are, respectively, 4 to 5 and 3 to 4 orders of magnitude above the normal plasma levels *in vivo*. Such levels are unlikely to ever occur, at least in non-(pro)renin synthesizing organs. Whether they do occur in (pro)renin-synthesizing tissues (e.g, in renal interstitial fluid) remains to be proven. The maximum plasma (pro)renin rises that have been described in humans are ≈50 to 100-fold for renin (but usually well below 10-fold) and 2- to 3-fold for prorenin [17, 29, 33]. Therefore, the phenotype that develops in response to (P)RR overexpression, per se (with no change in renin) [2,3], represents (P)RR effects that are renin-Ang system independent [13,14], as also evidenced by the lack of effect of such overexpression on plasma and tissue angiotensin levels. Vice versa, elevating plasma prorenin levels several hundred-fold in rodents might yield prorenin levels that do allow (P)RR-mediated Ang II generation, thus resulting in an Ang II–dependent phenotype (5,6). None of the transgenic rodent models with elevated prorenin levels display levels that are >1000-fold above normal [4–6,17,29], and, thus, it is not surprising that, in these models, in contrast with the models displaying (P)RR expression, neither renal fibrosis nor glomerulosclerosis occurred in an angiotensin-independent manner [4,6].

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Chapter 3

Urinary Renin and Angiotensinogen in Type 2 Diabetes: Added Value beyond Urinary Albumin?

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Abstract

Objective: Urinary levels of renin–angiotensin–aldosterone system (RAAS) components may reflect renal RAAS activity and/or the renal efficacy of RAAS inhibition. Our aim was to determine whether urinary angiotensinogen and renin are circulating RAAS-independent markers during RAAS blockade.

Methods: Urinary and plasma levels of angiotensinogen, renin, and albumin were measured in 22 patients with type 2 diabetes, hypertension, and albuminuria, during 2-month treatment periods with placebo, aliskiren, irbesartan, or their combination in random order in a crossover study.

Results: Aliskiren and irbesartan both increased plasma renin 3–4-fold, and above 10-fold when combined. Irbesartan decreased plasma angiotensinogen by approximately 25%, and no changes in plasma angiotensinogen were observed during the combination. Urine contained aliskiren at micromolar levels, blocking urinary renin by above 90%. Both blockers reduced urinary angiotensinogen, significant for irbesartan only. Combination blockade reduced urinary angiotensinogen even further. Reductions in urinary angiotensinogen paralleled albuminuria changes, and the urine/plasma concentration ratio of angiotensinogen was identical to that of albumin under all conditions. In contrast, urinary renin did not follow albumin, and remained unaltered after all treatments. Yet, the urine/plasma concentration ratio of renin was more than 100-fold higher than that of angiotensinogen and albumin, and approximately 4-fold reduced by single RAAS blockade, and more than 10-fold by dual RAAS blockade.

Conclusions: Aliskiren filters into urine and influences urinary renin measurements. The urine/plasma renin ratio, but not urinary renin alone, may reflect the renal efficacy of RAAS blockade. Urinary angiotensinogen is a marker of filtration barrier damage rather than intrarenal RAAS activity.

Introduction

There is an increasing interest in the intrarenal renin–angiotensin–aldosterone system (RAAS) and its role in progression of chronic kidney disease. Urinary levels of RAAS components have been suggested to reflect intrarenal RAAS activity [1–4], as angiotensinogen is thought to be synthesized in the proximal tubule and renin in the macula densa and collecting duct [5–7]. Since angiotensinogen has a high molecular weight (65 kD) similar to albumin (67 kD), circulating angiotensinogen will be filtered into urine only to a minimal extent [1]. In addition, this angiotensinogen is retrieved from the ultrafiltrate via megalin-dependent endocytosis [2]. Thus, if detected in urine, angiotensinogen is more likely to originate at renal tissue sites.

Renin has a lower molecular weight (48 kD), and a micropuncture study has observed that the renin concentration in the ultrafiltrate in Bowman's capsule is about 20% of the arterial plasma concentration [8]. Yet, the majority of this renin is reabsorbed, most likely in a megalin-dependent manner [2]. Therefore, high levels of urinary renin may also reflect renal RAAS activity, for example renin synthesis in the collecting duct.

Treatment of albuminuria includes RAAS blocking agents: renin inhibitors, angiotensin-converting enzyme inhibitors (ACEis), angiotensin II (Ang II) type 1 receptor blockers (ARBs), and mineralocorticoid receptor antagonists. Although RAAS blockade has been claimed to reduce the urinary angiotensinogen levels in patients with renal failure [3,4], evidence is still needed to demonstrate that this occurs fully independent of the reduction in urinary albumin, and is not the mere consequence of diminished glomerular filtration of large proteins.

In a previous study in 101 patients with hypertension and/or diabetes, we observed that RAAS blockade with either ACEi or ARBs increased the plasma renin concentration (PRC), and decreased urinary renin [9]. These opposite changes are in agreement with the concept that Ang II blocks renin release in the juxtaglomerular apparatus, and stimulates renin release in the collecting duct [6,7]. Consequently, the decrease in urinary renin might have been a reflection of the degree of RAAS blockade in the kidney, in particular, at the level of the collecting duct. Given the large diversity of the patients in that study, particularly with regard to treatment (also involving non-RAAS blockers that affect PRC, like [beta]-blockers and calcium antagonists), confirmation of these findings in a prospective study is mandatory.

Our aim was therefore to compare the urinary excretion of albumin, angiotensinogen, and renin during 2-month treatment periods with placebo, aliskiren, irbesartan, or their combination in a post-hoc analysis of a previously published clinical trial in patients with type 2 diabetes (T2D) [10]. Data on the plasma levels of these proteins have already been reported [10]. Our hypothesis was that urinary angiotensinogen, like albumin, is entirely plasma-derived, and that urinary renin is a better marker of renal RAAS activity than angiotensinogen. T2D patients are of particular interest, because of their paradoxical high responsiveness to RAAS blockade despite low PRC, resulting in the general belief of an activated renal tissue RAAS in such patients [11,12]. T2D patients also have elevated plasma levels of prorenin [13]. However, since we were unable to detect prorenin in urine samples of T2D patients in our previous study [9], we did not attempt to measure urinary prorenin in the present study.

Methods

Patients

This was a double-blind, randomized, crossover trial in accordance with the Declaration of Helsinki and Good Clinical Practice (GCP). The methods have been described in detail in the main publication [10]. After informed consent, patients underwent laboratory tests and evaluations of inclusion/exclusion criteria. A 1-month washout followed, in which all antihypertensive treatments were stopped. After washout, patients were randomized to 2 months of treatment with placebo, aliskiren 300 mg once daily, irbesartan 300 mg once daily, or the combination of the two, in random order. Patients with T2D, aged 30–80 years, were eligible for randomization with baseline urinary albumin excretion rate (UAER) greater than 100 mg/24 h, hypertension [office blood pressure (BP) >135/85 mmHg], and glomerular filtration rate (GFR) above 40 ml/min per 1.73 m2. Exclusion criteria included major cardiovascular disease, heart failure, glycated haemoglobin A1C more than 11%, and history of malignancy. At the end of each treatment period, patients collected three 24-h urine samples for assessment of geometric mean UAER. On the last day of each treatment period, 51Cr-EDTA GFR was measured [14].

Biochemical measurements

Of the 26 patients included in the original study, we had one urine sample available from all four treatment periods in 22 patients. Samples for the determination of PRC and plasma angiotensinogen were taken after 30 min of supine rest, and the plasma was frozen after centrifugation (–80°C). RAAS components were measured at the end of each treatment period. Urine was concentrated five-fold with Amicon Ultra-10 centrifugal filter devices (Millipore, Cork, Ireland) before applying the samples in the assay.

Plasma renin concentration was measured with an immunoradiometric kit (Renin III; Cisbio, Gif-sur-Yvette, France), with a detection limit of 1 pg/ml [15]. The urinary renin concentrations were sometimes below this detection limit. Therefore, urinary renin was measured with the more sensitive enzyme-kinetic assay (EKA), that is, by quantifying Ang I generation in the presence of excess sheep angiotensinogen [16]. The detection limit of the EKA is 0.05 ng Ang I/ml per h, and the Ang I-generating activities were converted to renin concentrations based on the fact that 1 ng Ang I/ml per h corresponds with 2.6 pg human renin/ml [17].

To determine whether aliskiren is present in urine of aliskiren-treated patients (potentially affecting the renin EKA), concentrated urine samples of five aliskiren-treated and five placebo-treated patients were incubated with 330 pg/ml recombinant human renin (obtained from the Cisbio kit, Gif-sur-Yvette, France) in the EKA. An aliskiren standard curve was generated by adding aliskiren in increasing concentrations (1 nmol/l–10 μmol/l) to urine samples of three healthy male volunteers prior to their concentration and application in the EKA in the presence of 330 pg/ml renin. The amount of Ang I generated per tube was 607 ± 10 pg in the control urine without aliskiren. Following the addition of 1, 10, 100, 1000, and 10 000 nmol/l aliskiren to

urine, the generated Ang I amount decreased to, respectively, 558 ± 7 (mean ± SEM), 277 ± 12, 31 ± 1, 1 ± 1, and 3 ± 1 pg/tube. In the urine of the five placebo-treated patients, the amount of Ang I generated per tube (660 ± 31 pg) was identical to that in the control urines without aliskiren. In the urine of the aliskiren-treated patients only 17 ± 11 pg/ml Ang I was generated. This indicates that, on average, the aliskiren levels in the urine samples of aliskiren-treated patients was greater than 0.1 μmol/l, that is sufficient to block renin by more than 90% [18]. Consequently, the EKA would underestimate the renin levels in urine of aliskiren-treated patients by more than 90%, and thus, in such samples (following concentration), the urinary renin levels were additionally measured by immunoradiometric assay as described above.

Plasma angiotensinogen was measured as the maximum quantity of Ang I that was generated during incubation with excess recombinant renin [19]. The detection limit of this assay is 0.50 pmol/ml. Urinary angiotensinogen was measured with a commercial angiotensinogen ELISA (IBL International, Hamburg, Germany), with a detection limit of 0.01 pmol/ml.

Statistics

Values are expressed as mean ± SD, mean ± SEM, or geometric mean and range. Levels below the detection limit were taken to be equal to the detection limit. Wilcoxon matched pairs test was used to compare the effect of treatment versus placebo. Linear regression was used to investigate associations between RAAS components and albumin, as well as for analysis of associations between treatment-induced changes in parameters. A two-tailed P-value of less than 0.05 was considered statistically significant.

Results

Baseline characteristics of the 22 patients who completed all treatment periods can be seen in Table 1. Table 2 provides the renin, angiotensinogen, and albumin levels in plasma and urine. Aliskiren and irbesartan increased PRC ($P < 0.001$), and the increase was even larger when treatments were combined. Irbesartan decreased plasma angiotensinogen ($P < 0.001$), whereas aliskiren neither alone, nor in combination affected plasma angiotensinogen. No drug affected plasma albumin.

Aliskiren and irbesartan reduced urinary angiotensinogen similarly, although significantly, only for irbesartan ($P = 0.02$), possibly due to the fact that irbesartan also reduced plasma angiotensinogen by approximately 25%. With combined treatments, the reduction in urinary angiotensinogen was even larger ($P < 0.001$). The findings on angiotensinogen fully paralleled the effects of treatment on urinary albumin. Yet, despite the massive changes in PRC, treatment did not significantly affect urinary renin. When applying the EKA to the samples of the aliskiren-treated patients, the renin levels calculated from the generated amount of Ang I were 0.1 ±

0.1 and 0.2 ± 0.1 ng/l, that is, more than 10 times lower than the levels measured by immunoradiometric assay. This demonstrates that urine contains aliskiren at levels that block renin by more than 90%, that is, levels that are in the micromolar range [18]. Clearly, therefore, as in plasma during aliskiren treatment, only a direct renin assay provides a true estimate of the renin concentrations in urine of aliskiren-treated patients.

Table 1. Baseline characteristics of 22 patients with type 2 diabetes mellitus, hypertension, and albuminuria

Parameter	
Ethnicity (Caucasian %)	100
Male, *n* (%)	16 (73)
BMI (kg/m^2)	32.2 ± 5.3
GFR (ml/min per 1.73 m^2)	88.3 ± 24.7
Albuminuria (mg/24 h)	345 (102–1023)
Office SBP (mmHg)	137.6 ± 16.2
Office DBP (mmHg)	81.4 ± 11.4
24-h SBP (mmHg)	142.5 ± 12.3
24-h DBP (mmHg)	74.4 ± 7.4
Total cholesterol (mmol/l)	3.7 ± 0.74
HbA_{1c} (%)	8.2 ± 1.4

GFR, glomerular filtration rate; HbA_{1c}, glycated haemoglobin A_{1c}.
Data are mean ± SD or geometric mean (range).

Urinary levels were corrected for changes in plasma levels, by calculating the urine/plasma concentration ratio [9]. The urine/plasma ratios for angiotensinogen and albumin were identical under all circumstances, whereas the urine/plasma ratio for renin was more than 100-fold higher (Table 2). The urine/plasma angiotensinogen concentration ratio paralleled the urine/plasma albumin concentration ratio in all groups (Fig. 1), whereas urinary renin was unrelated to urinary albumin (Fig. 2). Importantly, the urine/plasma ratio for renin decreased approximately four-fold during single RAAS blockade, and more than 10-fold during dual RAAS blockade (Table 2).

Table 2. Plasma and urinary renin, angiotensinogen (Aogen), and albumin levels and their urine/plasma (U/P) concentration ratios × 100% in 22 patients with type 2 diabetes following a 2-month treatment period with placebo, aliskiren, irbesartan, or their combination.

Parameter	Placebo	Aliskiren	Irbesartan	Aliskiren + Irbesartan
$[Renin]_{plasma}$ (ng/l)	29 (6–180)	111 (18–1024)*	80 (9–868)*	335 (42–1926)*
$[Renin]_{urine}$ (ng/l)	1.6 (0.03–24)	1.8 (0.03–13)	1.3 (0.04–14)	2.0 (0.46–62)
U/P ratio renin (×100%)	5.6 (0.1–119)	1.6 (0.1–70)**	1.7 (0.1–28)**	0.6 (0.1–13)*
$[Aogen]_{plasma}$ (nmol/l)	969 (772–2686)	956 (500–2502)	719 (317–2480)*	899 (605–2564)
$[Aogen]_{urine}$ (nmol/l)	0.074 (0.030–0.497)	0.058 (0.005–0.311)	0.036 (0.005–0.483)***	0.023 (0.005–0.270)*
U/P ratio Aogen (×100%)	0.008 (0.001–0.055)	0.006 (0.001–0.037)	0.005 (0.001–0.067)	0.003 (0.001–0.031)*
$[Albumin]_{plasma}$ (g/l)	40 (37–44)	41 (37–62)	41 (36–46)	41 (32–46)
$[Albumin]_{urine}$ (mg/l)	5.4 (3.6–7.8)	4.8 (2.7–6.5)**	4.5 (2.7–6.5)*	4.2 (2.6–6.2)*
U/P ratio Albumin (×100%)	0.013 (0.008–0.020)	0.012 (0.007–0.016)**	0.011 (0.006–0.015)**	0.010 (0.007–0.015)*

Data are geometric mean and range.
*$P < 0.001$.
**$P < 0.01$.
***$P = 0.02$ versus placebo.

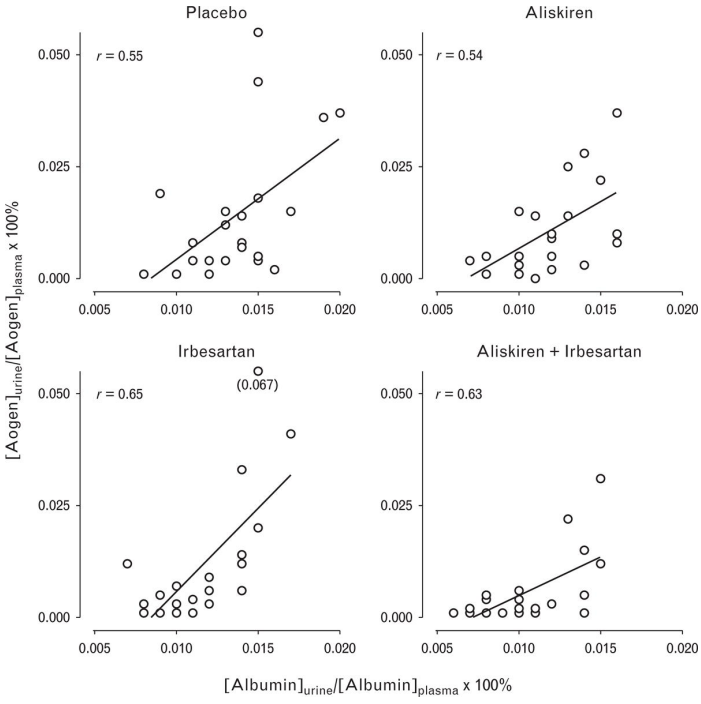

Figure 1. Correlations between urine/plasma ratio of albumin versus angiotensinogen (Aogen) during placebo and during aliskiren, irbesartan, and combination treatment (P = 0.013, 0.007, <0.001, and 0.003, respectively).

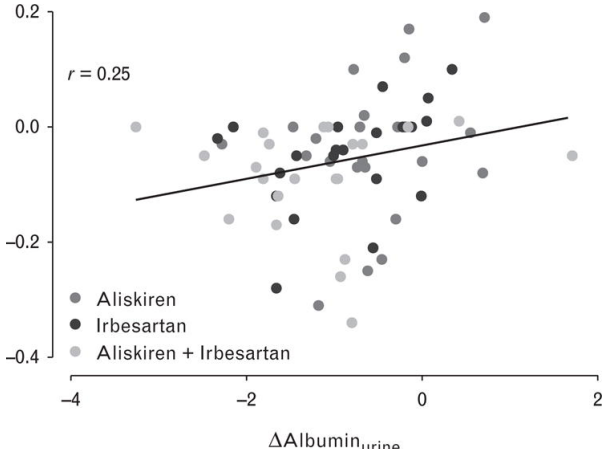

Figure 2. Correlation between changes in urinary angiotensinogen concentration (ng/l) and urinary albumin concentration (mg/l), during the three treatment periods combined (P = 0.050).

Finally, the treatment-induced change in urinary angiotensinogen paralleled the change in urinary albumin (Fig. 3). Again, this was not true for urinary renin (Fig. 4).

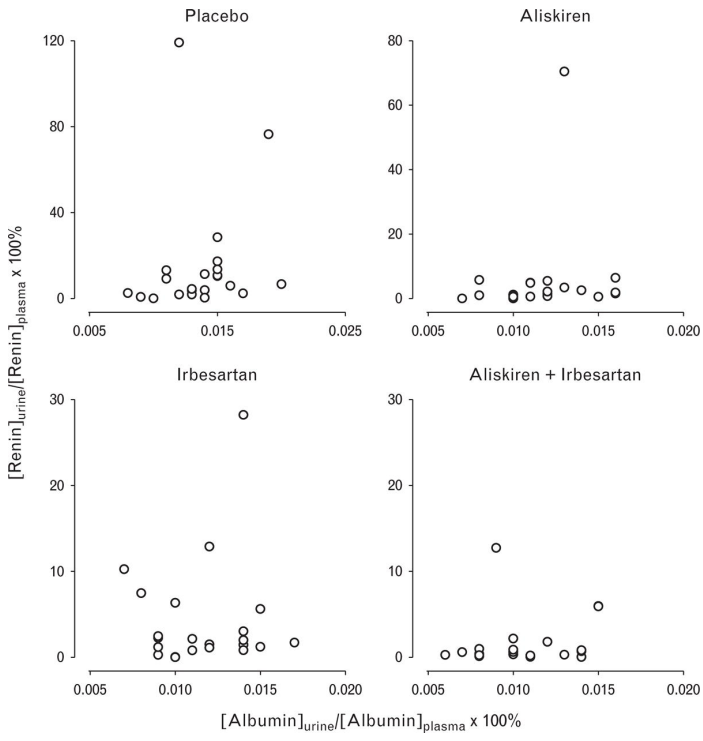

Figure 3. Correlations between urine/plasma ratio of albumin versus renin during placebo and during aliskiren, irbesartan, and combination treatment (P = NS in all cases).

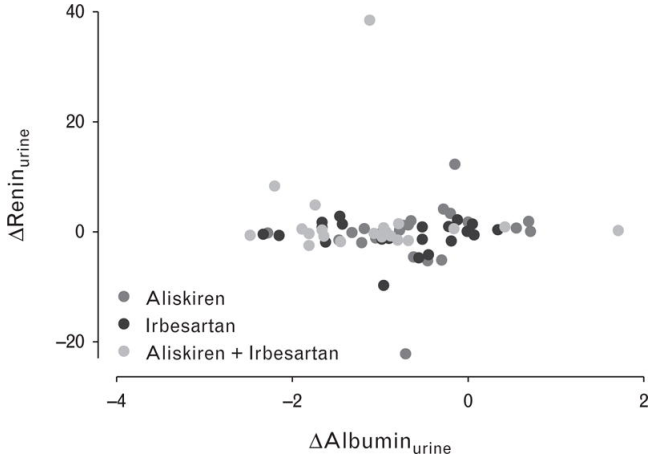

Figure 4. Correlation between changes in urinary renin concentration (ng/l) and urinary albumin concentration (mg/l), during the three treatment periods combined (P = NS).

Discussion

In this study, we did not find that urinary renin levels in T2D patients were affected by RAAS blockade with a renin inhibitor, an ARB, or their combination. We found that, during aliskiren treatment, urine contained aliskiren levels that were capable of blocking renin by more than 90%. On the basis of previous studies investigating the inhibitory capacity of aliskiren [18], this implies that the urinary aliskiren levels are in the micromolar range, that is, comparable to the plasma levels of aliskiren during treatment with a dose of 300 mg aliskiren/day [20,21]. Finally, we observed that under all conditions, even during placebo, the urine/plasma concentration ratios of albumin and angiotensinogen were highly correlated, and that the RAAS blocker-induced changes in urinary albumin paralleled those in albumin.

Angiotensinogen has a size and mass that is comparable to that of albumin. Our data therefore suggest that both proteins filter to the same degree from plasma into urine, and that measurements of urinary angiotensinogen provide the same information as measurements of urinary albumin. This contradicts a series of findings by Kobori et al.[4,22,23], who suggested that urinary angiotensinogen exclusively reflects renal RAAS activity, in particular angiotensinogen production, at the level of the proximal tubulus.

A recent study by Nakano et al.[1] utilized multiphoton microscopy to visualize the glomerular permeability of circulating angiotensinogen in mouse and rat kidney. These authors did find increased sieving of angiotensinogen with increasing level of glomerulosclerosis, but not to a degree that would explain the rise in urinary angiotensinogen in this condition. In fact, when infusing human angiotensinogen in rats developing glomerulosclerosis, its glomerular permeability correlated with the urinary albumin levels, but not the urinary angiotensinogen levels. Moreover, its sieving coefficient was four times lower than that of albumin. Yet, in our current study the urine/plasma ratios of albumin and angiotensinogen were identical, and for both proteins, it could be calculated that their urinary levels during placebo treatment were approximately 0.01% of those in plasma. These levels decreased in parallel to even lower values during RAAS blocker treatment, suggesting a common origin, that is, filtration from circulating blood. To explain the apparent discrepancy between our data and those of Nakano et al., it should be taken into account that the latter authors infused human angiotensinogen as a bolus injection over a period of 2–3 min through the carotid artery. Consequently, no steady state had been reached. Since angiotensinogen, like renin, diffuses into the interstitium [16,24], reaching interstitial levels that are comparable to those in plasma [25], a substantial amount of the infused human angiotensinogen would be expected to diffuse into the interstitial space in the first few hours after injection, thus potentially resulting in urinary levels of human angiotensinogen that are far below 0.01% of the plasma levels, that is, levels that are not detectable with the applied assay (the same assay we used). This could explain the virtual absence of human angiotensinogen in rodent urine in these studies.

Interestingly, in mice, Nakano et al.[1] detected urinary mouse angiotensinogen levels of approximately 2 ng/90 min versus approximately 2000 ng/ml in plasma. In rats developing glomerulosclerosis, the urinary rat angiotensinogen levels were approximately 30 ng/90 min at young age, and these levels increased to approximately 100 ng/90 min at older age. Given the urinary volume of rodents of at most a few millilitres per day, this implies that in such animals, the endogenous angiotensinogen levels in urine are above two orders of magnitude higher than our patients (Table 2). Nakano et al. claim this urinary angiotensinogen to originate in the tubules. Paradoxically, it has recently been shown that proximal tubule-specific angiotensinogen knockout mice have renal angiotensinogen and Ang II levels that are similar to those in control mice [5], both under normal conditions and following podocyte injury. Thus, even if angiotensinogen is synthesized in the kidney, it has no functional role, that is, it does not contribute to renal angiotensin production. Our data now clearly demonstrate that, in T2D patients with nephropathy, urinary angiotensinogen levels are not only more than 100 times below the urinary levels of endogenous angiotensinogen in rats and mice, but also correlate closely with urinary albumin. We conclude that in humans, the release of nonfunctional angiotensinogen into urine from proximal tubular synthesis sites does not occur, and that all angiotensinogen in urine, like albumin, is blood-derived. Clearly, therefore, urinary angiotensinogen, like albumin, is a marker of glomerular permeability, and the measurement of both proteins offers the same information. Interestingly, Alge et al.[26] recently identified urinary angiotensinogen and albumin as the two strongest biomarkers in patients with acute kidney injury, predicting the need for renal replacement therapy, and they subsequently confirmed the prognostic predictive power of urinary angiotensinogen in 97 patients who underwent cardiac surgery. Unfortunately, they did not verify the prognostic power of urinary albumin, despite the many studies that support such a role [27–29].

Our findings on urinary renin are more difficult to interpret. In a previous study in patients with hypertension and/or diabetes, we observed that RAAS blockade with either ACEi or ARBs modestly increased PRC [from 11 to 17 ng/l (geometric means of n = 101)], and decreased urinary renin from 1.6 to 0.7 ng/l [9]. In contrast, urinary renin in the present study did not decrease during single RAAS blockade, and if anything, tended to increase during combination blockade. A striking difference compared with the previous study is that PRC during placebo treatment in the present study was 29 ng/l (i.e. three times higher) and rose almost 12-fold during dual RAAS blockade (Table 2). In both studies, urinary renin was unrelated to urinary albumin, and renin's urine/plasma ratio was more than 100 times higher than that of angiotensinogen and albumin. The latter may relate, at least in part, to the smaller molecular weight of renin, facilitating its glomerular filtration.

There are at least three possible explanations of the current findings. First, urinary renin may originate exclusively in the kidney, from a source that is regulated independently from the juxtaglomerular apparatus, and that apparently does not respond to RAAS blockade. This would contradict our previous study. Second,

urinary renin may be entirely blood-derived. This should normally result in some correlation with other plasma-derived components, like albumin and angiotensinogen. Since this was not the case, renin reabsorption in the proximal tubule may vary [18], and/or plasma-derived prorenin may have been converted to renin following its glomerular filtration. The latter would also explain why urine does not contain prorenin [9]. Third, and perhaps most likely, urinary renin may originate both in the kidney and blood. Under low PRC conditions, urinary renin would then be primarily kidney (collecting duct)-derived, and might even decrease following RAAS blockade, as observed in our previous study. However, if such blockade massively increases PRC (like in the present study), glomerular filtration of blood-derived renin will come into play and mask the reduction in collecting duct-derived renin. To solve this issue, studies in collecting duct-specific prorenin knockout rodents are required. However, irrespective of the underlying mechanism, the current study clearly shows that urinary renin per se cannot be considered as a marker that exclusively reflects the degree of renal RAAS activity. Yet, the renin urine/plasma ratio may help to determine the degree of renal RAAS blockade, given the decrease in this ratio depending on the number of RAAS blockers (single versus dual treatment; Table 2).

In summary, this analysis presents findings from a study with only 22 diabetic patients and a limited follow-up. Yet, it is a single-center, double-blind, randomized intervention study, with standardized measurements and repeated 24-h urine collections as important strengths. Our study sheds light on the origin of urinary angiotensinogen and renin, and demonstrates that both parameters by themselves cannot be used to assess the degree of renal RAAS blockade, at least in patients with T2D and signs of glomerular damage. Urinary angiotensinogen is a marker of filtration barrier damage rather than intrarenal RAAS activity, and as such is expected to have the same predictive power as urinary albumin. Urinary renin did not change during RAAS blockade, despite more than 10-fold rises in plasma renin and a reduction in albuminuria. Yet, the renin urine/plasma ratio may accurately reflect the degree of renal RAAS blockade. Given the activated renal RAAS in diabetic patients, our observations should now be extended to a nondiabetic control group with microalbuminuria, to assess the consequences of RAAS blockade in primary glomerular diseases without renal RAAS activation.

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Chapter 4

(Pro)renin Receptor Is Required for Prorenin-Dependent and -Independent Regulation of Vacuolar H^+-ATPase Activity in MDCK.C11 Collecting Duct Cells

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Abstract

Prorenin binding to the prorenin receptor ((P)RR) results in non-proteolytic activation of prorenin, but also directly (i.e., independent of angiotensin generation) activates signal transduction cascades that can lead to upregulation of profibrotic factors. The (P)RR is an accessory protein of the vacuolar-type H^+-ATPase (V-ATPase) and is required for V-ATPase integrity. In addition, in collecting duct cells prorenin-induced activation of the extracellular signal-regulated kinase depends on V-ATPase activity. However, whether prorenin binding to the (P)RR directly regulates V-ATPase activity is as yet unknown. Here, we studied the effect of prorenin on plasma membrane V-ATPase activity in Madin Darby Canine Kidney clone 11 (MDCK.C11) cells, which resemble the intercalated cells of the collecting duct. Prorenin increased V-ATPase activity at low nanomolar concentrations, and the V-ATPase inhibitor bafilomycin A1, but not the angiotensin II type 1 and 2 receptor blockers irbesartan and PD123319, prevented this. Increased, but not basal V-ATPase activity was abolished by siRNA depletion of the (P)RR. Unexpectedly, the putative peptidic (P)RR blocker Handle Region Peptide (HRP) also increasedV-ATPase activity in a (P)RR-dependent manner. Finally, [Arg8]-vasopressin-stimulated V-ATPase activity and cAMP production were also abolished by (P)RR depletion. Our results show that in MDCK.C11 cells, the (P)RR is required for prorenin-dependent and –independent regulation of V-ATPase activity.

Introduction

The (pro)renin receptor ((P)RR) confers full catalytic activity to bound prorenin by a non-proteolytic mechanism, resulting in renin-angiotensin system (RAS) activity [1]. Binding of prorenin to the (P)RR also directly engages signal transduction cascades independent from Ang II formation, for example activation of extracellular signal-regulated kinase 1/2 (Erk1/2) and subsequent upregulation of profibrotic factors like transforming growth factor β1 (TGF-β1), collagen and fibonectin [1-4]. Plasma prorenin, but not renin, is increased in diabetes mellitus, and the elevated prorenin levels correlate well with the severity of diabetic nephropathy [5]. Hence, a local prorenin-(P)RR interaction resulting in fibrosis and inflammation, directly or by increased RAS activity, appears an attractive mechanism that may contribute to end-organ damage in patients with diabetes mellitus. However, given the nanomolar affinity of prorenin for the (P)RR, as opposed to its picomolar levels in blood plasma, it is currently uncertain whether such interaction truly occurs in vivo. The (P)RR also binds renin, but given its ≈5-fold lower affinity [6, 7] and the ≈10-fold lower levels of renin as compared to prorenin [8], in vivo renin-(P)RR interaction is highly unlikely, since this would require an almost 100.000-fold rise in renin.

The (P)RR was previously identified as an accessory protein of the vacuolar-type H^+-ATPase (V-ATPase) [9]. V-ATPases are multisubunit transporters that consist of a Vo-domain that translocates protons across membranes, driven by the hydrolysis of ATP by the V1-domain. V-ATPase are expressed in virtually all cell types and are mainly found in intracellular compartments, where they play important roles in organellar acidification, receptor-mediated endocytosis, protein trafficking and degradation by acidifying intracellular compartments [10, 11]. Depletion of the (P)RR in several cell types results in downregulation of V-ATPase subunits, causing impaired acidification of intercellular compartments, autophagy defects and eventually cell death [12-14]. In some cell types, V-ATPases are also abundantly present at the plasma membrane, for example in the kidney in the intercalated cells (ICs) of the collecting duct, where they are important for urine acidification. Concomitantly, mutations in renal-specific V-ATPase subunits are associated with distal renal tubular acidosis [15]. The (P)RR is also strongly expressed in the ICs of the collecting duct and colocalizes with V-ATPase at the luminal membrane [3]. Interestingly, both (P)RR expression and prorenin secretion are increased in the collecting duct in diabetes mellitus [16-19], raising the possibility that locally sufficiently high prorenin levels may be reached to bind to the (P)RR.

Advani et al. [3] highlighted a functional interaction between the (P)RR and V-ATPases in collecting duct cells, by showing that the (P)RR and V-ATPase activity are required for prorenin-induced activation of Erk1/2 in Madin-Darby Canine Kidney (MDCK) cells. Whether V-ATPase activity itself is regulated by prorenin-(P)RR interaction, however, is unknown. Here we demonstrate that prorenin stimulates V-ATPase activity via the (P)RR in cells of the MDCK clone 11 (MDCK.C11), which specifically resemble the ICs of the collecting duct [20]. In addition, our data reveal an unexpected agonist function of the putative (P)RR blocker handle region peptide (HRP) in stimulating V-ATPase activity. Finally, we show that the (P)RR is also required for vasopressin-induced V-ATPase activity and cAMP accumulation, independent of prorenin-binding.

Methods

Reagents and Solutions

The 2',7'-bis(2-carboxylethyl)-5(6)-carboxyfluorescein ester (BCECF-AM) was purchased from Life Technologies (Bleiswijk, The Netherlands). Bafilomycin A1 was purchased from Millipore (Amsterdam, The Netherlands). 5-(N-Ethyl-N-isopropyl)-amiloride (EIPA) and Schering 28080 (Sch-28080) were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands). Recombinant human prorenin was a kind gift from Dr. W. Fischli (Actelion Pharmaceuticals Ltd, Allschwil, Switzerland). Rat HRP (RILLKKMPSV-OH) and human HRP (IFLKRMPSI-OH) were synthesized by Biosyntan (Berlin, Germany). [Arg8]-vasopressin (AVP) was obtained from Sigma Aldrich, and was used as positive control for V-ATPase activity measurement. Irbesartan, PD123319 and forskolin were obtained from Sigma Aldrich.

Cell culture and siRNA transfection

Madin-Darby Canine Kidney (MDCK) cells clone 11 (MDCK.C11) were a kind gift from Dr. Michael Gekle (Julius-Bernstein-Institut für Physiologie, Jena, Germany). Cell culture of MDCK.C11 cells was performed as described elsewhere [20]. In brief, cells were cultured in MEM (Life Technologies) supplemented with 10% FBS (Thermo Fisher Scientific, Aalst, Belgium), 2 mM L-glutamine (Life Technologies) and 100 U/ml penicillin, 100 mg/ml streptomycin (Lonza, Basel, Switzerland) at 37 °C in humidified air with 5% CO2. Cells were passed once a week and medium was changed every two days. For V-ATPase activity measurements, 3×104 cells were seeded in each well in 24-wells-plates, and for immunoblotting 1×105 cells were seeded in each well in 6-wells-plates. After reaching 80-90% confluence, cells were serum starved for 24 hours before performing the pHi recovery experiment. To knockdown (P)RR expression with siRNA, cells were transfected using Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol and used 48 hours after siRNA transfection. Stealth siRNA against the (P)RR was designed based on the canine (P) RR sequence (GenBank accession number XM_003435497) and obtained from Life Technologies. The sequences are: GGGCUACCAUUAUGGUGAUGGUG (sense) and UUCACCAUCACCAUAAUGGUAGCCC (anti-sense). Silencer® Negative control No.1 siRNA was obtained from Life Technologies, and applied at same concentration as siRNA against (P)RR.

V-ATPase activity

V-ATPase activity was determined as the rate of recovery of the intracellular pH (dpHi/dt) from an NH4Cl-induced acid load as described [21, 22], with modifications using a plate reader to measure fluorescence in cells loaded with the pH sensitive dye BCECF-AM. Buffers used are listed in Table 1. Cells were washed, and incubated in Buffer I containing 2 μM BCECF-AM for 20 min at 37 °C. Cells were then washed with Buffer I and incubated for 5 min in NH4Cl-buffer at 37 °C. After NH4Cl incubation, cells were rapidly washed and incubated with Buffer II at 37 °C. Where indicated, prorenin and HRP were added to NH4Cl buffer 1 min prior to the end of acid loading, and were present in the Buffer II during pHi recovery. To block other pHi-regulating transporters, cells were incubated in the absence of Na+, HCO3- and CO2, and in the presence of the K+-H+-ATPase inhibitor Sch-28080 (100 μM) and Na+-H+ exchanger inhibitor EIPA (5 μM). Fluorescence ratios were measured by alternatively exciting the dye at 495 and 440 nM while monitoring the emission at 530 nM, using a SpectraMax GEMINI EM plate reader (Molecular Devices, Sunnyvale, CA, U.S.). Fluorescence ratios were converted to pHi by calibrating cells with 10 μM nigericin (Invitrogen) in 105 mM KCl as described [23, 24]. V-ATPase activity was expressed as dpHi/dt over a range of pHi (6.45-6.7) of wells with similar acid loading. In some experiments, 200 nM of the V-ATPase inhibitor Bafilomycin A1 (Baf A1) was added to determine V-ATPase-independent activity, or 1 μM irbesartan and 1 μM PD123319 were added to block angiotensin type 1 and 2 receptors during the NH4Cl pulse and pHi recovery.

Immunoblotting

Cells were washed twice with ice cold PBS and lysed at 4 °C in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 3 mM KCl, 1 mM EDTA, 1% Triton X-100, complete protease inhibitors (Roche, Woerden, The Netherlands), phosphate inhibitors (Sigma Aldrich), pH 7.4. Lysates were centrifuged at 1000×g for 5 min at 4 °C, and protein concentrations in supernatants measured with a BCA assay (Pierce, Etten-Leur, The Netherlands). Equal amounts of proteins (20 μg) were separated by SDS-PAGE and transferred to PVDF membranes (Milipore) for immunoblotting. Blots were probed with antibodies against (P)RR/ATP6AP2 (1:1000, Sigma- Aldrich), β-actin (1:50000, Millipore), ATP6V1B1/B2 (1:2000 in 5% BSA, Santa Cruz), ATP6Voa2 (1:1000, Abnova), ATP6Vod2 (1:1000, Abcam) and ATP6Voa4 (1:7500)[25]. Bound antibody was detected by enhanced chemiluminescence (Pierce) using horse-radish peroxidase conjugated secondary antibodies (1:3000, Biorad, Veenendaal, The Netherlands).

Intracellular cAMP measurement

Prior to stimulation, growth medium were removed and MDCK cells were incubated in serum free MEM at 37 °C in the presence of 250 μM of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX, Sigma) for 30 min to prevent cAMP hydrolysis . Cells were then treated with 100 nM AVP or 1 μM forskolin (Sigma) in the presence of IBMX for 15 min. Cells were immediately put on ice, and washed twice with ice-cold PBS-CM (PBS containing 1 mM CaCl2, and 0.1 mM MgCl2, pH 7.4) and lysed in 150 μl 0.1 N HCl on ice. Lysates were centrifuged at 1000×g for 5 min at 4 °C. Intracellular cAMP concentrations were determined by cAMP direct EIA kit (ITK Diagnostics, Uithoorn, The Netherlands) following manufacturer's protocol, and corrected for the protein concentrations in supernatants as determined with the BCA assay.

Statistics

Data are presented as mean ± SEM. One-way ANOVA followed by Bonferroni correction was performed for comparison of more than two groups. All other data were tested for significance using unpaired Student's t-test. $P<0.05$ was considered significant.

Results

Prorenin stimulates V-ATPase activity in MDCK.C11 cells

Prorenin-induced Erk1/2 phosphorylation in MDCK cells depends on V-ATPase activity [3]. To determine whether V-ATPase activity itself is regulated by prorenin, we used cells of the MDCK.C11 clone, which resemble the ICs of the collecting duct and have high plasma membrane V-ATPase activity [20], and measured V-ATPase activity as the rate of recovery of the intracellular pH (dpHi/dt) from an NH4Cl-induced acid load. To minimize the activity of other pHi-regulating transporters, we allowed the cells to recover in the absence of CO2/HCO3- and Na+ to block sodium-bicarbonate cotransporter, anion exchanger, and sodium-proton exchanger (NHE) activity in Buffer II (Table 1), and in the presence of SCH28080, to block K^+/H^+-ATPase

activity and EIPA to block any residual NHE activity. As a positive control, we also stimulated cells with 100 nM [Arg8]-vasopressin (AVP). Addition of 10 nM prorenin to the recovery buffer induced a moderate (as compared to the AVP control) but significant increase in dpHi/dt, compared to vehicle control (Figures 1A and 1B). The increase in dpHi/dt was due to V-ATPase activity, since bafilomycin A1 (Baf A1), a potent and selective inhibitor of V-ATPase activity, decreased dpHi/dt in both control and prorenin-treated cells to similar residual activities (Figure 1B). Prorenin also increased V-ATPase activity in the presence of the angiotensin type 1 and 2 receptor blockers irbesartan and PD123309, indicating that the prorenin-induced increase in V-ATPase activity is independent of the formation of Ang II (Figure 1C). Since 10 nM prorenin is a supraphysiological concentration, we also tested lower concentrations and found that prorenin increases V-ATPase activity in a dose-dependent manner (Figure 1D), however, only with 1 nM and 10 nM prorenin the increases were significant. Although from our data we cannot conclude that 10 nM prorenin is a saturating concentration, sigmoidal curve fitting by setting the response of 100 nM AVP as maximal response gave an EC50 of ~0.5 nM, which is around 10-fold lower than the Kd of prorenin binding to the (P)RR [1, 6, 7, 26]. Collectively, these data show that in MDCK.C11 cells, prorenin stimulates V-ATPase activity, independent of Ang II.

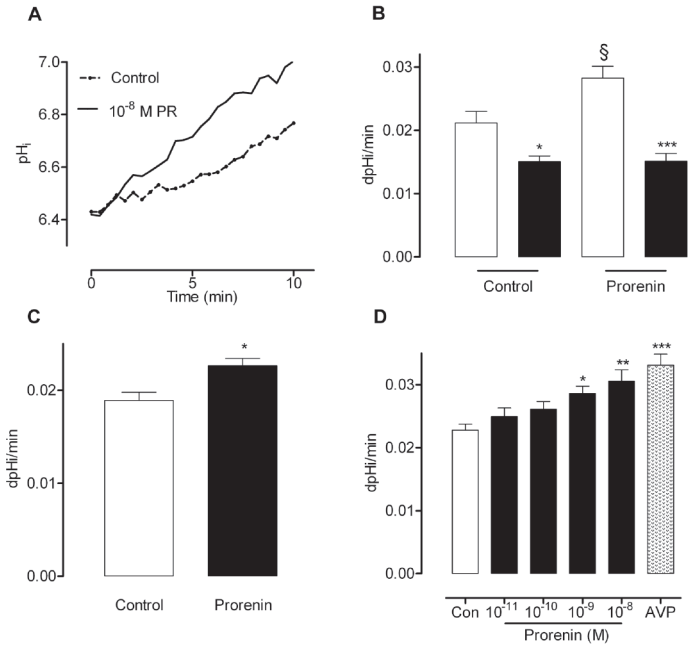

Figure 1. ***pHi recovery measurements in MDCK.C11 cells show that prorenin (PR) stimulates V-ATPase activity in a dose dependent manner.*** (A) Example of pHi recovery curve. The slope of the initial linear phase was taken as a measure for the rate of recovery (dpHi/dt). 10 nM prorenin increased the rate of recovery. (B) Baf A1-insensitive dpHi/dt is comparable in the presence or absence of 10 nM prorenin, confirming that the prorenin-induced increase in dpHi/dt is due to increased V-ATPase activity (n=10-13). §: 10 nM compared to vehicle control, $p<0.01$. Open bar: absence of Baf A1; Closed bar: Presence of Baf A1.(C) V-ATPase activity in the presence of 1 μM irbesartan and 1 μM PD123309 shows that prorenin increases V-ATPase activity independent of Ang II (n=9). (D) Prorenin stimulates V-ATPase activity in a dose dependent manner. 100 nM AVP was applied as positive control. (n=11-17). *: $p<0.05$;***: $p<0.001$.

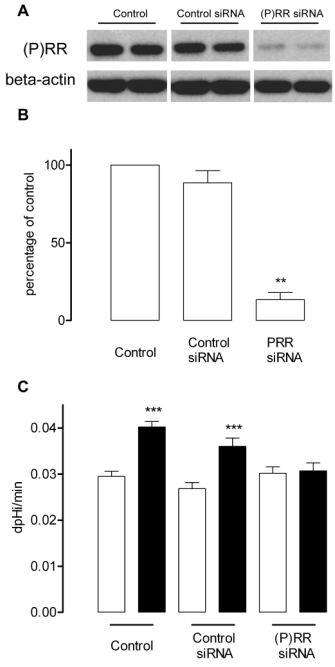

Figure 2. ***Effect of siRNA against the canine (P)RR on (A and B) (P)RR expression and (C) dpHi/dt shows that prorenin increases V-ATPase activity via the (P)RR.*** (A) Representative western blot of lysates from untransfected, and control - or (P)RR siRNA-treated MCDK.C11 cells. Samples from two independent experiments in duplicate were loaded for blotting. For illustrative purpose, two lanes were cut from the original blot for both control, control siRNA and (P) RR siRNA. (B) (P)RR protein, corrected for β-actin, in lysates of control or (P)RR siRNA transfected cells and expressed as percentage of untransfected control show that the (P)RR is efficiently knocked down. Results from three independent experiments in duplicate. **:p<0.01 compared to untransfected and control siRNA transfected cells. (C) (P)RR knockdown abolished prorenin-induced, but not basal V-ATPase activity in MDCK.C11 cells. Open bars indicates cells treated with vehicle while closed bar indicates cells treated with 10 nM prorenin. (n=17-22). **:p<0.01, ***: p<0.001 compared to matched vehicle treated cells.

The (P)RR is required for prorenin-stimulated V-ATPase activity

To test whether prorenin-stimulation of V-ATPase activity is mediated by the (P)RR, we designed siRNAs against the canine (P)RR to knock down (P)RR expression in MDCK.C11 cells. Immunoblotting lysates of transfected cells with (P)RR antibodies revealed that our siRNAs reduced (P)RR protein levels by more than 80% (Figures 2A and 2B). Knocking down (P)RR expression did not alter basal V-ATPase activity, but fully abolished the prorenin–stimulated increase in V-ATPase activity, whereas the stimulated activity was unaffected in untransfected cells or cells transfected with control siRNA (Figure 2C). These data confirm that in MDCK.C11 cells prorenin stimulates V-ATPase activity in a (P)RR-dependent manner.

The peptidic (P)RR blocker HRP stimulates V-ATPase activity

We next asked whether prorenin-induced V-ATPase activity can be blocked by the peptidic (P)RR blocker HRP. Previous study using immobilized (P)RR showed that HRP binds to the (P)RR with nanomolar affinity and can partially displace bound prorenin [7, 27]. We therefore tested whether HRP could block the prorenin-induced increase in V-ATPase activity. We found that 1 μM rat HRP (rHRP) did not prevent the prorenin-induced increase in dpHi/dt. Surprisingly, 1 μM rHRP alone also increased dpHi/dt in MDCK.C11 cells to a similar level as 10 nM prorenin (Figure 3A). The Baf A1-insensitive rate of pHi-recovery was similar in control and rHRP-treated cells, confirming that rHRP specifically increases V-ATPase activity (Figure 3B). The increase in V-ATPase activity was dose-dependent (Figure 3C), reaching significance at 100 nM and 1 μM rHRP. Sigmoidal curve fitting by setting the response of 100 nM

AVP as maximal response gave an EC50 of ~130 nM. Human HRP (hHRP) also dose-dependently increased V-ATPase activity, although the increase was less pronounced and only significant at 1 μM hHRP (data not shown). rHRP-stimulated but not basal V-ATPase activity was fully blocked in MDCK.C11 cells transfected with siRNA against the (P)RR, but not in cells transfected with control siRNA or in nontransfected cells (Figure 3D). These data show that like prorenin, HRP stimulates V-ATPase activity in a (P)RR-dependent manner, revealing an unexpected agonist function of HRP.

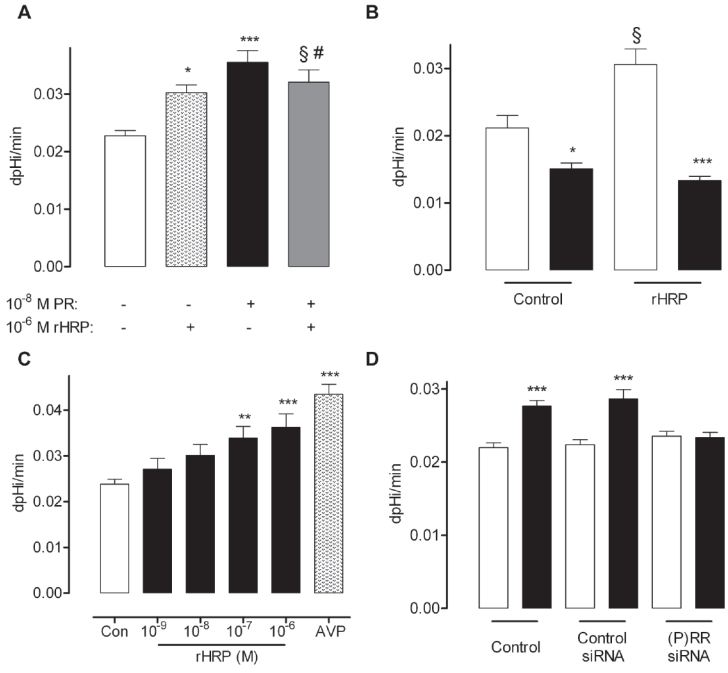

Figure 3. ***pHi recovery measurements in MDCK.C11 cells show that rHRP is not an inhibitor of prorenin-induced increase in V-ATPase activity (A), but stimulates V-ATPase activity itself (A-D).*** (A) 1 μM rHRP does not significantly block the increase dpHi/dt induced by 10 nM prorenin, and alone increases dpHi/dt (n=15-19). §: 10-8 M PR and 10-6 M rHRP treated compared to control, p<0.01; #:10-8 M PR and 10-6 M rHRP treated compared to 10-8 M PR treated, ns. (B) Baf A1- insensitive dpHi/dt is comparable in the presence or absence of 1 μM rHRP, comfirming that the rHRP-induced increase in dpHi/dt is due to increased V-ATPase activity. Open bar indicates cells treated with vehicle while closed bar indicates cells treated with Baf A1 (n=3-13). §: 10-6 M rHRP treated compared to control, p<0.01. (C) rHRP stimulates V-ATPase activity in a dose dependent manner (n=6-10). (D) (P)RR knock-down abolishes HRP-induced V-ATPase activity. Cells were transfected as in figure 2. Open bar indicates cells treated with vehicle while closed bar indicates cells treated with 1 μM HRP (n=10-21). *: p<0.05; ***: p<0.001.

(P)RR knockdown decreases the abundance of selective V-ATPase subunits

In podocytes, cardiomyocytes and mouse embryonic fibroblasts, where V-ATPases are restricted to intracellular compartments, (P)RR depletion selectively decreases the abundance of subunits of the Vo domain of the V-ATPase [12, 13].

To test whether in ICs, where V-ATPases are also present at the plasma membrane, the (P)RR is required for V-ATPase integrity as well, we immunoblotted lysates from siRNA transfected MDCK.C11 cells with antibodies against several V-ATPase subunits. We found that knocking down (P)RR expression decreased the abundance of the a2, but not the kidney-specific a4 and d2 subunits of the Vo domain (Figures 4A and 4B). In addition, expression of the B2 and the kidney-specific B1 subunit of the V1 domain were also unaffected by (P)RR depletion. These data suggest that in intercalated cells, (P)RR expression is required for the abundance of some but not all Vo domain subunits consistent with preserved V-ATPase activity in (P)RR depleted cells.

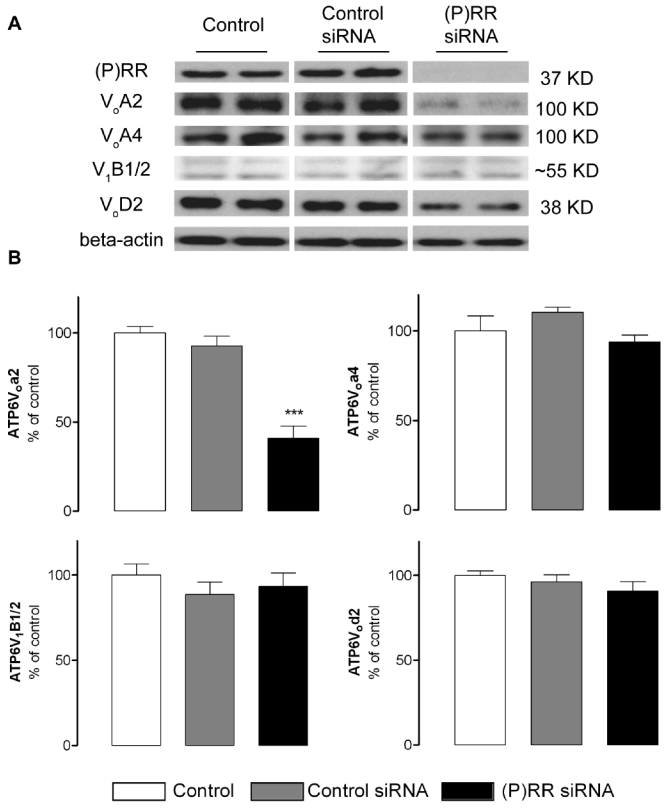

Figure 4. ***Knocking down (P)RR expression decreases the abundance of the Voa2 subunit but not the Voa4, Vod2 and V1B1/2 subunits.*** Samples from two independent experiments in duplicate were loaded for blotting. For illustrative purpose, two lanes were cut from the original blot for both control, control siRNA and (P)RR siRNA. (A) Representative western blot of lysates from untransfected, and control- or (P)RR siRNA-treated MCDK.C11 cells. (B) Protein abundance of V-ATPase subunits, corrected for β-actin, in lysates of control or (P)RR siRNA transfected cells and expressed as percentage of untransfected control show that only abundance of the Voa2 subunit was significantly decreased by knocking-down (P)RR expression (upper left). Results from three independent experiments in duplicate. ***: $p<0.001$.

The (P)RR is required for AVP-induced V-ATPase activity and cAMP generation

We demonstrated that the (P)RR is required for prorenin- and HRP-stimulated V-ATPase activity. To determine whether the (P)RR is required for V-ATPase stimulation by other ligands as well, we tested the effect of (P)RR depletion on AVP induced V-ATPase activity in MDCK.C11 cells. In nontransfected cells and cells transfected with the negative control siRNA, 100 nM AVP induced an significant increase in Baf A1-sensitive dpHi/dt (Figure 5A) that was abolished in cells transfected with siRNA against the (P)RR (Figure 5B), confirming that the (P)RR is required for AVP-stimulated V-ATPase activity. In MDCK and isolated ICs, AVP increases the production of cAMP [20, 28, 29], an important regulator of V-ATPase activity [30, 31]. cAMP/PKA pathway regulates V-ATPase activity and apical accumulation in kidney cells [32, 33]. We therefore tested if (P)RR depletion also impairs AVP-stimulated cAMP production.

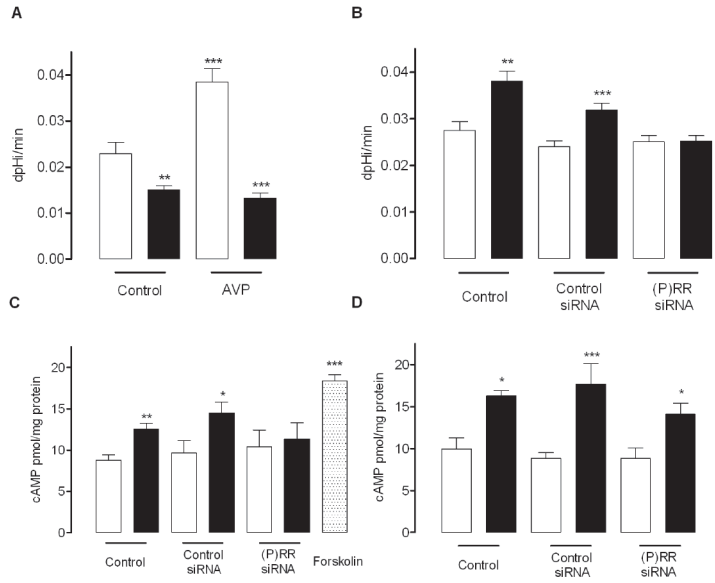

Figure 5. ***pHi recovery and cAMP measurements in MDCK.C11 cells show that AVP-induced increase in V-ATPase activity and cAMP production depends on the presence of the (P)RR.*** (A) 100 nM AVP significantly increased dpHi/dt, which is inhibited by Baf A1. Baf A1-insensitive dpHi/dt is comparable in the presence or absence of 100 nM AVP, confirming that AVP induced increase in dpHi/dt is due to increased V-ATPase activity. Open bar indicates cells treated with vehicle while closed bar indicates cells treated with Baf A1 (n=10-14). **: p<0.01; ***: p<0.001. (B) (P)RR knockdown abolishes AVP-induced V-ATPase activity. Open bar indicates cells treated with vehicle while closed bar indicates cells treated with 100 nM AVP (n=10-18). **: p<0.01; ***: p<0.001. (C) AVP significantly increases intracellular cAMP accumulation, which is abolished in cells transfected with (P)RR siRNA but not control siRNA-treated cells. 1 μM forskolin was used as positive control. Levels of cAMP were corrected for protein concentration, and represented two independent experiments in triplicate. Open bar indicates cells treated with vehicle while closed bar indicates cells treated with 100 nM AVP. *: p<0.05; **: p<0.01; ***: p<0.01. (D) 1 μM forskolin significantly increases cAMP, which is not affected by (P)RR siRNA treatment. Levels of cAMP were corrected for protein concentration and represented two independent experiments in triplicate. Open bar indicates cells treated with vehicle while closed bar indicates cells treated with 1 μM forskolin. *: p<0.05; ***: p<0.01.

Stimulation of nontransfected or negative control siRNA transfected MDCK.C11 cells with 100 nM AVP or 1 μM forskolin as positive control significantly increased cAMP production (Figure 5C), whereas in cells transfected with siRNA against the (P)RR, the AVP-induced increase in cAMP production was abolished. To investigate whether (P)RR depletion impairs vasopressin receptor-signaling or directly affects adenylyl cyclase activity, we tested the effect of (P)RR knockdown on the cAMP response to forskolin. 1 uM forskolin significantly increased cAMP production, and this effect was not affected by (P)RR siRNA (Figure 5D), indicating that vasopressin receptor-signaling rather than adenylyl cyclase activity itself is impaired by (P)RR depletion. Collectively, these data show that in MDCK.C11 cells, the (P)RR is also required for AVP-induced V-ATPase activity and signaling, independent from (pro)renin.

Discussion

Our study shows that prorenin stimulates V-ATPase activity dose-dependently in MDCK.C11 cells. This stimulation was abolished by knocking down the (P)RR but not by adding AT receptor blockers. These findings show that prorenin-(P)RR binding directly stimulates V-ATPase activity, independent from prorenin activity. Furthermore, our study shows that the putative peptidic (P)RR antagonist HRP also stimulates V-ATPase activity via the (P)RR, which suggests that for certain responses, binding of HRP to the (P)RR may have stimulatory rather than inhibitory effects.

Similar to most in vitro prorenin responses, a significant increase in V-ATPase activity required supraphysiological concentrations of prorenin, >100 times higher than plasma prorenin concentrations in normal subjects (~5 pM) [34]. Even under pathological conditions, for example in diabetes mellitus, plasma prorenin levels are at most 5-10–fold increased [5, 35], which is still insufficient to stimulate the V-ATPase.

Renin and prorenin are released from the juxtaglomerular (JGA) cells of the kidney, and such release may also occur in the collecting duct, particularly under pathological conditions [36]. For example, the collecting duct displays increased renin expression in Ang II-infused rats [37] and might be the source of the increased prorenin levels in diabetic Munich-Wistar rats [16]. Renal (P)RR expression levels are also highest in the collecting duct, with the protein predominantly found at the plasma membrane of the ICs [3] and (P)RR expression increases even further in diabetic nepropathy [18, 38]. Hence, particularly in the diabetic kidney, local luminal prorenin concentrations in the collecting duct are likely to be high enough to increase V-ATPase activity via the (P)RR. In several cell types, including mesangial [2] and collecting duct cells [3], (pro)renin induces Erk1/2 phosphorylation, resulting in the production of profibrotic factors [2, 4]. Advani et al [3] found that in MDCK cells, (pro)renin-induced Erk1/2 phosphorylation was blocked by Baf A1, arguing for a role for V-ATPase in (pro)renin-induced signaling in the collecting duct. In addition, in proximal tubular epithelial cells TGF-β1-induced epithelial-mesenchymal

transition, an important contributor to renal fibrosis, is partially blocked by Baf A1 [39], further indicating that V-ATPase activity can contribute to renal damage.

The kidney plays an essential role in the maintenance of acid-base homeostasis by reabsorbing filtered bicarbonate and secreting acid and base equivalents. V-ATPases are abundantly expressed at the plasma membrane across different segments of the nephron, where they account for 45-50% of total H+ secretion [40]. The highest densities are found in the ICs of the collecting duct, where V-ATPases acidify the final urine [41]. Could prorenin contribute to V-ATPase activity and urine acidification? Patients with type 2 diabetes have an increased risk of nephrolithiasis caused by uric acid stones [42-44]. Decreased urine pH reduces uric acid solubility and is a major cause of stone formation. Accordingly, type 2 diabetes is associated with a lower urine pH and this association persists after correction for body mass index and acid intake [42]. Since ammonium ions are a major determinant of urine pH, decreased renal ammonia generation caused by insulin resistance in type 2 diabetes has been suggested as a likely cause of the lower urine pH [43]. However, patients with type 2 diabetes had a similar increase in ammonium secretion after an oral acid load compared to healthy subjects, indicating that other mechanisms must be involved [42]. Although increased secretion of organic acids caused by altered glucose metabolism is an obvious candidate, it may be tempting to determine whether increased prorenin levels correlate with decreased urine pH, and whether transgenic models that overexpress the Renin gene have a lower urine pH that is resistant to RAS blockade.

How does the (P)RR regulate V-ATPase activity? V-ATPases are regulated by several mechanisms including association/disassociation of the V1 and Vo domain, recycling of V-ATPase containing vesicles to and from the plasma membrane and altered coupling efficiency between ATP hydrolysis and proton translocation [15, 32, 45-47]. Because the (P)RR is associated with the V-ATPase, a mechanism by which prorenin-(P)RR association induces a conformational change that somehow increases V-ATPase activity, for example by altering the coupling efficiency, would appear the simplest explanation. However, our data showed that knocking-down (P)RR expression not only abolished prorenin-, but also AVP-induced V-ATPase activity, which argues for a more fundamental role for the (P)RR in V-ATPase regulation. MDCK.C11 cells have been well-characterized by Gekle et al. [20] and resemble ICs. Such cells predominantly express V1 receptors [48].. However, V2 receptor expression has also been found in type A intercalated cells in isolated rat, mouse and human kidneys [49], and thus MDCK.C11 cells might similarly express functional V2 receptors. Although V2 receptors are classically linked to adenylyl cyclase activation and cAMP formation, such formation has also been reported after V1 receptor stimulation [50, 51]. Thus, in MDCK.C11 cells, AVP may rapidly stimulate V-ATPase activity via both V1 - and V2 receptors, involving increases in intracellular Ca2+ and/or cAMP [30, 52]. In outer medullary collecting duct (OMCD) ICs, cAMP stimulates V-ATPase activity and insertion into the apical membrane [31], and blockade of cAMP-dependent protein kinase blocks aldosterone-stimulated V-ATPase activity [53]. Ca2+ is also required for

aldosterone and Ang II stimulation of V-ATPase activity in outer medullary collecting duct ICs, likely via protein kinase C (PKC) activation [53, 54]. We showed that in MDCK.C11 cells, AVP increased cAMP production depending on the presence of the (P)RR, which may indicate that impaired cAMP production in the (P)RR-depleted cells prevents insertion of V-ATPase complexes into the plasma membrane. This would also explain why only stimulated (and not basal) V-ATPase activity is affected in (P)RR knock-down cells, similar to the effects of cAMP-dependent protein kinase inhibition [30, 53]. Most likely, analogous to the (P)RR role in Wnt signaling [55], the (P)RR functions as an adaptor protein for vasopressin receptor-signaling. Indeed, our observation rules out that the (P)RR directly affects adenylyl cyclase activity.

Previous studies showed that (P)RR depletion results in downregulation of Vo subunits [12-14], which would predict a decrease in basal activity. We found, however, that of the Vo subunits tested, only the abundance of the Voa2-subunit was decreased. There are four isoforms of the Voa subunit (a1-a4), which are all expressed differently in tissues. The Voa4 is the most abundant and dominant isoform in the kidney, especially in the cortical collecting duct, and is predominantly expressed at the plasma membrane of the kidney [56-58]. This isoform was unaffected by (P)RR depletion, which may explain why (P)RR depletion did not affect basal V-ATPase activity. Whether prorenin uses similar pathways to stimulate V-ATPase activity is as yet unclear; renin increased neither cAMP nor Ca2+ in human mesangial cells that overexpress the (P)RR [1].

Finally, stimulation of V-ATPase activity by aldosterone and Ang II depends on Erk1/2 activation [54]. Prorenin activates Erk1/2 in MDCK cells even at picomolar concentration, which suggests that different pathways may exist. Despite differences in amino acid compositions, both rat and human HRP increased in V-ATPase activity at high concentrations, although hHRP was less potent, and the rHRP-induced increase depended on the presence of the (P)RR. The stimulatory effect of HRP is in contradiction with the inhibition of renin/prorenin-induced Erk1/2 phosphorylation in growing rat mesangial cells [59] and mouse endothelial cells [60] and beneficial effects of HRP infusion on end-organ damage in disease models [19, 60, 61]. Other studies, however, found that HRP had no affect on prorenin-induced Erk1/2 activation in human vascular smooth muscle cells and monocytes [6, 62], did not improve nephrosclerosis in Goldblatt rats, and even counteracted the beneficial effects of renin inhibition in spontaneously hypertensive rats [63]. Some observers also reported stimulatory effects of HRP on Erk1/2 phosphorylation [64, 65], causing neuronal and glia injury in the retina, agreeing with our findings that, depending on the model and concentrations used, HRP can act as a (P)RR agonist. Although some studies even question binding of HRP to the (P)RR in cells [62, 66], our data showed that HRP stimulation of V-ATPase activity is dependent on the (P)RR, and thus a unifying concept is that HRP acts as a partial agonist.

In conclusion, we showed the (P)RR is required for both prorenin and prorenin-independent stimulation of V-ATPase activity in collecting duct cells via the (P)RR. Due to high concentrations of prorenin required, we speculate this is unlikely to normally occur in vivo, unless under pathologic conditions in which local prorenin levels are highly increased. We also showed that HRP stimulates V-ATPase dependent on the (P)RR, which preaches caution for the use of and interpretation of results found with his putative (P)RR blocker.

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Chapter 5

Tandem-affinity Purification of the (Pro)renin Receptor Reveals a Functional Interaction with Sortilin-1

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In Preparation

Abstract

(Pro)renin binding to the (pro)renin receptor [(P)RR] activates both angiotensin-dependent and –independent signaling pathways. Recent findings indicate that the (P)RR has additional functions independent from (pro)renin binding. The (P)RR is required as an accessory protein of the vacuolar H^+-ATPase (V-ATPase), for maintaining V-ATPase integrity and Wnt signaling. These findings reveal that protein-protein interactions are important for (P)RR signaling. To discover additional (P)RR-interacting proteins, we purified human (P)RR from HEK293 cells using a tandem affinity purification method and identified co-purifying proteins with mass spectrometry. Our method was vindicated by the presence of V-ATPase subunits in the (P)RR-associated profile. In addition, we found several candidate (P)RR-binding proteins, with functions in signaling and lipid metabolism. Using co-immunoprecipitations, we confirmed the interaction between the (P)RR and a protein in our profile, sortilin-1, a multiligand receptor that plays important roles in neuronal viability and signaling, and LDL metabolism. Furthermore, we found that the (P)RR and sortilin-1 mutually regulate protein abundance. These results reveal a potential novel role for the (P)RR in neuronal and lipid biology, and show that our approach can be useful to obtain a better understanding of the functions of the (P)RR.

Introduction

The (pro)renin receptor ((P)RR) is a 350-amino acid protein that can bind both renin and prorenin (collectively known as (pro)renin) [1-5]. The (P)RR induces conformational changes in bound prorenin, leading to non-proteolytic activation of prorenin. In several cell models, binding of (pro)renin to (P)RR also directly activates signaling pathways independent from the formation of angiotensin (Ang) II, for example, activation of the extracellular signal-regulated kinase 1/2 (Erk1/2) and subsequent upregulation of profibrotic factors such, as transforming growth factor-β1 (TGF-β1), collagen and fibronectin [1, 6-10]. How the (P)RR relays signals to downstream effectors is as yet unknown. The (P)RR is a single transmembrane protein with a large N-terminal extracellular (pro)renin-binding domain and a short intracellular C-terminal domain, and has no homology with known receptors and lacks obvious protein-protein interaction domains. Protein-protein interactions are likely important for signaling, as the promyelocytic leukemia zinc finger (PLZF) can directly bind the (P)RR to downregulate (P)RR expression [11], whereas in vascular smooth muscle cells (VSMCs), prorenin stimulates Erk1/2 and Akt phosphorylation via cross-activation of the epidermal growth factor receptor (EGFR) [12]. However, since the (pro)renin concentrations that are required for signaling are several orders of magnitude higher than that found in human subjects and animal models, even in high-(pro)renin pathologies, such as diabetes mellitus, or in prorenin transgenic animals [13], it is unlikely that (pro)renin-(P)RR interactions occur in vivo, except maybe in tissues where abundant cell surface (P)RR and (pro)renin secretion are co-localized.

Instead, recent findings have indicated novel functions for the (P)RR, independent from (pro)renin binding. The (P)RR was previously identified as an accessory protein of the vacuolar-type H+-ATPase (V-ATPase) [14] V-ATPases are multisubunit complexes that consists of V0 and V1 domains. They are expressed in virtually all cell types and play an important role in acidifying intracellular compartments [15, 16]. The (P)RR is indispensable for V-ATPase integrity, as depletion of the (P)RR in several cell types results in decreased protein levels of V-ATPase subunits and impaired acidification of intracellular compartments [17-19]. The functional interaction between the (P)RR and the V-ATPase also regulates signaling, both (pro)renin-dependent and –independent [20]. Moreover, the (P)RR is required for prorenin-dependent and –independent stimulation of V-ATPase activity in collecting duct cells in vitro [21]. Furthermore, several recent studies have demonstrated that the (P)RR is required for Wnt signaling. In the canonical Wnt/β-catenin pathway, Wnt molecules bind to a complex of Frizzled (Fz) receptors and low-density lipoprotein receptor-related protein receptors (LRP) 5/6, resulting in internalization of this complex into signalosomes. The signalosomes require acidification by V-ATPases for downstream Wnt signaling, which depends on the association of the (P)RR with the V-ATPase and Fz/LRP complex [22]. In the non-canonical Wnt/Planar Cell Polarity (PCP) pathway, the (P)RR also binds Fz-receptors and is required for the asymmetrical localization of Fz-receptors during epithelial polarization [23, 24]. Finally, The (P)RR may mediate signaling by other receptors as well. A (P)RR mutation that results in 50% expression of a truncated form (Δ4-(P)RR) was found in a family of patients with X-linked mental retardation [25]. Expression of Δ4-(P)RR in neuronal PC-12 cells inhibits nerve growth factor (NGF)-induced Erk1/2 phosphorylation [26].

To discover (P)RR signaling partners and potential novel functions, we used an unbiased proteomics approach. For this, we purified the human (P)RR from HEK293 cells using a tandem affinity purification (TAP) method and identified co-purifying proteins by mass spectrometry. Our method was vindicated by the presence of several V-ATPase subunits in the complex and revealed putative (P)RR-interacting proteins with functions in signaling and lipid metabolism. From the proteins identified, we confirmed the interaction between the (P)RR and sortilin-1, a multiligand sorting receptor that contains a VSP10 domain and two endolysosomal sorting motifs [27], which plays important roles in regulating neuron viability and functions, and LDL metabolism. These results show that our proteomics approach can be a useful tool to understand the functions of the (P)RR.

Methods

Plasmid generation

The pCeMM-NTAP vector was used to express TAP-tagged human (P)RR [28]. The TAP-tag consists of two IgG binding domains of protein G, separated from streptavidin binding protein (SBP) by a consensus cleavage site for the Tobacco Etch Virus protease. The vector further contains an internal ribosomal entry site (IRES) to co-express green fluorescent protein (GFP) of a bicistronic messenger, which was used to confirm transfection and expression of the plasmid. The signal peptide of human (P)RR, as determined by the SignalP server (http://www.cbs.dtu.dk/services/SignalP-3.0/) was inserted as a primer duplex (sense primer: 5'-P-AATTCGCTG-TGTTTGTCGTGCTCCTGGCGTTGGTGGCGGGTGTTTTGGGGAACG-3', antisense primer: 5'-P-AATTCGTTCCCCAAAACACCCGCCACCAACGCCAG-GAGCACGACAAACACAGCG-3') in the EcoRI-site immediately upstream from the TAP-tag. The remainder of the human (P)RR sequence was amplified by PCR (forward primer: 5'-CTCGAGTATATTAAAATCACCAGGGTCTGTTG, reverse primer: 5'-GCGGCCGCTCAATCCATTCGAATCTTCTGG-3') and inserted into the XhoI and NotI site. The correct sequence of the insert was confirmed by sequencing. Construction of the pCeMM-NTAP-h(P)RR plasmid is illustrated in Figure 1.

Cell culture and transfection

HEK293 cells were cultured with DMEM medium (Lonza, Basel, Switzerland) supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin (Lonza) at 37 °C in humidified air with 5% CO2. Culture medium was replaced every two days, and cells were passed when they reach 80-90% confluence. To express NTAP-h(P)RR, HEK293 cells were plated in culture medium without antibiotics in T-75 culture flask a night before transfection. At the time of transfection, cells were about 80-90% confluence. Cells were transfected with Lipofectamine 2000 following manufacture's protocol. For transfecting one flask of T-75 HEK293 cells, 24 µg plasmid DNA was used. Transfection efficiency and plasmid expression were checked by GFP signal 24 hours after transfection. At the time checked, about 90% cells were GFP positive. Cells were harvested 48 hours after transfection. siRNA against Sort-1 was purchased from Life Technologies (Ambion® Select, S224557, Bleiswijk, The Netherlands). siRNA against (P)RR was synthesized by Life Technology with sequence GACAGUGUUGCAAAUUCCAUUCACU (sense), and AGUGAAUGGAAUUUGCAACACUGUC (anti-sense). HEK293 cells were transfected with 40 nM siRNAs using Lipofectamine 2000 following manufacture's protocol.

Tandem affinity purification

Cells were washed twice with ice cold PBS, and lysed on ice with lysis buffer (20 mM Tris-HCl, 3 mM KCl, 1 mM EDTA, 1% NP-40, 150mM NaCl, 50 mM NaF, 1 mM Na3VO4, pH 7.4) supplemented with complete protease inhibitors cocktailTM (Roche, Woerden, The Netherlands). For affinity purification, 10 flasks of T-75 were used for

each condition. Cell lysates were collected and cleared by centrifugation twice at 1500g for 10 min at 4 °C. Then cell lysates were mixed with 1 mL prewashed IgG sepharose beads (GE healthcare, Luxemburg), and incubated on a rotating platform for 2 hours at 4°C. Beads were collected by centrifugation at 1000g for 3 minutes at 4°C. Beads were washed three times with lysis buffer containing protease inhibitors and three times with TEV buffer containing protease inhibitors. TEV protease (700 U) was added to beads resuspended in 1 mL TEV buffer containing protease inhibitors. TEV protease and TEV buffer (20x) were purchased from Life technologies. Beads were incubated over night on rotating platform at 4 °C. After incubation, supernatants were collected by centrifugation at 1000g for 3 minutes at 4°C. Then, 100 µl prewashed streptavidin-agrose beads (Life Technologies) were added to the supernatant, and incubated on a rotating platform for 1h at 4 °C. After incubation, streptavidin-agrose beads were collected by centrifugation at 1000g for 3 minutes at 4°C, and washed five times with lysis buffer containing protease inhibitor. Protein complexes were eluted from the beads by adding 80 ul of NuPage® LDS sample loading buffer, and incubating at 95 °C for 5 min. Proteins were separated by SDS-PAGE on 4-20% Novex® Bis-Tris gradient gels using MOPS buffer (Life Technologies) and visualized by colloidal coomassie blue staining (Life Technologies) according to the manufacturer's protocol.

Liquid Chromotography/Tandem Mass Spectrometry analysis

Complete SDS-PAGE gel lanes were cut into ~1 mm slices using an automatic gel slicer and subjected to in-gel reduction with dithiothreitol, alkylation with chloroacetamide and digestion with sequencing graded porcine trypsin (Promega) as described [29]. Nanoflow LC-MS/MS was performed on an 1100 series capillary LC system (Agilent Technologies) coupled to an LTQ Orbitrap XL mass spectrometer (Thermo Scientific), operating in positive mode and equipped with a nanospray source. Peptide mixtures were trapped on a ReproSil C18 reversed phase column (Dr Maisch GmbH; column dimensions 1.5 cm × 100 µm, packed in-house) at a flow rate of 8 µl/min. Peptide separation was performed on ReproSil C18 reversed phase column (Dr Maisch GmbH; column dimensions 15 cm × 50 µm, packed in-house) using a linear gradient from 0 to 80% B (A = 0.1 % formic acid; B = 80% (v/v) acetonitrile, 0.1 % formic acid) in 70 min and at a constant flow rate of 200 nl/min using a splitter. The column eluent was directly sprayed into the ESI source of the mass spectrometer. Mass spectra were acquired in continuum mode; fragmentation of the peptides was performed in data-dependent mode by CID. Peak lists were automatically created from raw data files using the Proteome Discoverer (version 1.3; Thermo). The Mascot search algorithm (version 2.2, MatrixScience) was used for searching against the Uniprot database (release unihuman_2012_02_cont.fasta, taxonomy: all entries). The peptide tolerance was set to 10 ppm and the fragment ion tolerance was set to 0.8 Da. A maximum number of 2 missed cleavages by trypsin were allowed and carbamidomethylated cysteine and oxidized methionine were set as fixed and variable modifications, respectively. The Mascot score cut-off value for a positive protein hit was set to 40.

Immunoprecipitation and immunoblotting

For co-immunoprecipitations (CoIPs) of (P)RR with sortilin-1, HEK293 cells were grown in T-75 flasks until 80-90% confluence. Cells were washed twice with ice-cold PBS and lyzed in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM NaF, 1 mM Na3VO4, 5 mM EDTA, 0.8% NP-40 and complete protease inhibitors cocktailTM, pH 7.5.) on ice for 15 minutes. Cell lysates were cleared by centrifugation at 1000g for 5 minutes at 4 °C. Lysates were normalized for protein content, determined with the bicinchoninic acid (BCA) assay (Pierce, Etten-Leur, The Netherlands), and used directly for immunoblotting or used for immunoprecipitating the (P) RR or sortilin-1. For immunoprecipitations, the lysates were precleared with 50 µL prewashed ProG Dynabeads® (Life Technologies) on a rotating platform for 1 hour at 4 °C. 1 mg proteins were incubated on a rotating platform for 1 hour at 4 °C with 9 µg anti-h(P)RR antibodies (GeneTex, Irvine, USA), anti-sortilin-1 antibodies (Abcam, Cambridge, England) or non-specific rabbit IgGs (Life Technologies)] as control, coupled to 50 µL Protein-A Dynabeads. After washing, the immunocomplexes were eluted from the beads by adding 50 µl NuPage® LDS sample loading buffer (Life Technologies), and incubating at 95 °C for 5 min. Proteins from total lysates or immunocomplexes were separated by SDS-PAGE and transferred to PVDF membranes for immunoblotting. Blots were probed with antibodies against the (P)RR (anti-ATP6AP2, 1:1000, Sigma), anti-Sortilin-1 (1:1000, BD Bioscience, Breda, The Netherlands), anti-β-actin (1:50,000, Millipore, Amsterdam, The Netherlands), and anti-LRP6 (1:1000, Millipore). Bound antibody was detected using an enhanced chemilumiscence kit (Pierce).

RNA isolation and qPCR

Cells cultured in 6-well-plate were washed once with ice-cold PBS, and lyzed with 1 mL Trizol (Life Technologies). Total RNA was isolated following manufacturer's protocol and reversely transcribed to cDNAs using the QuantiTect Reverse Transcription kit (Qiagen, Venlo, The Netherlands). The resulting cDNAs were amplified in 40 cycles (denaturation at 95°C for 10min, thermo cycling at 95°C for 15s, annealing at 60°C for 1 min) with a STEP-ONE cycler (Life Technologies) using SYBR green PCR master mix (Life Technologies). Primers for detecting (P)RR expression were purchased from Qiagen (QT00078260). Primers for Sort1 were synthesized by Life Technologies with sequences: TGACCGGAAAGTTGGGGTTC (forward), and AGTACACGTTTCCTCGGCAG (reverse). Gene expressionwas relatively quantified by the ΔΔCT method by normalizing for β-actin gene expression. The primer set for detecting human β-actin was synthesized by Life Technologies with sequences: CTCCCTGGAGAAGAGCTACG (forward) and GAAGGAAGGCTGGAAGAGTG (reverse).

Statistic analysis

Data are presented as means ± SEM. One-way ANOVA followed by the Bonferroni correction was performed for comparison of more than two groups. P values of <0.05 were considered significant.

Results

Purification of (P)RR-associated proteins

The mechanisms by which the (P)RR signals to downstream pathways is largely unknown, but likely involves protein-protein interactions. To identify putative (P)RR-interacting proteins, we expressed full length human (P)RR tagged with the TAP-epitope in HEK293 cells. The TAP-epitope consists of two IgG-binding domains of protein G and streptavidin-binding protein, separated by a Tobacco Etch Virus (TEV) consensus site, which allows cleavage of the tagged protein from the IgG-affinity resin after the first purification step with recombinant TEV. We choose to tag at the N-terminus, since the C-terminal domain consists of a small and highly conserved intracellular tail where putative interactions could be sterically hindered by the presence of a large tag. To allow appropriate protein targeting and expression, the signal peptide of the (P)RR was placed in front of the tag. The construct we used expresses NTAP- (P)RR of a bicistronic messenger that also encodes GFP to monitor expression (Figure 1). We initially established HEK293 cell lines that stably express either NTAP or NTAP-h(P)RR by co-transfection with a plasmid containing the neomycine selection marker, however, NTAP-(P)RR was weakly expressed compared to transiently transfected cells and tended to decrease over time (data not shown). We therefore decided to use HEK293 cells that transiently expressed NTAP-(P)RR. The NTAP-(P)RR protein or the NTAP only as control were purified by sequential purification on IgG-sepharose and streptavidin-agarose and proteins in the final eluate resolved on 4-20% SDS-PAGE gels. Colloidal coomassie blue staining of the gels revealed a single band around 15 kDa in eluates from cells expressing the NTAP control plasmid (Figure 2), which is the expected size of the NTAP tag after TEV-cleavage. Eluates from cells overexpressing NTAP-(P)RR revealed a band with comparable intensity to the NTAP band around 50 kDa, which is the expected size of TEV-cleaved NTAP-(P)RR, as well as several additional bands that were not found in the control lane. We subjected entire SDS-PAGE lanes from two independent NTAP-(P)RR purifications and matching controls to in-gel trypsin digestion, followed by LC/MS-MS analysis. We found 49 and 205 proteins that specifically co-purified with NTAP-(P)RR in purifications 1 and 2 respectively. For a stringent profile, we only selected proteins that were found in both purifications (40 in total), which we grouped by molecular function (Table 1). We made an exception for the V-ATPase subunits and accessory proteins Vod, ATP6AP1/Ac45 and VMA21, which were only found in one purification, but included based on the known interaction of the (P)RR with the V-ATPase complex [[14, 22], Table 1]. The presence of the (P)RR/ATP6AP2 as well as several components of the V-ATPase in our profile vindicated our method and suggested that NTAP-(P)RR was functional. However, in spite of the absence in our control purifications, many proteins from our profile are abundantly present in the CRAPome (denoted by asterisks in Table 1), a recently published database of contaminants in currently 343 affinity purification-mass spectrometry datasets [[30], www.crapome.org], which suggests that a substantial number of our hits are false positives, most notably proteins involved in mitochondrial transport, protein folding and modification, DNA binding and cytoskeletal organization.

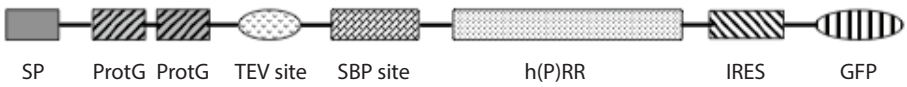

Figure 1. ***Schematic of the NTAP-h(P)RR construct. Human (P)RR was tagged with the TAP-epitop at the N-terminal side.*** The signal peptide (SP) of the human (P)RR was inserted at N-terminus before the TAP-tag. The TAP-tag contains two IgG-binding domains from ProtG, and Streptavidin-binding protein, separated by a TEV cleavage site. An IRES is present to express GFP of a bicistronic messenger to monitor expression of the construct.

The (P)RR is associated with sortilin-1 in HEK293 cells

To further validate our method, we selected a protein from the NTAP-(P)RR-associated profile for further analysis. We selected sortilin-1 for technical reasons and possible physiological relevance. Sortilin-1 was found in two independent purifications with high confidence. Sortilin-1, also known as neurotensin receptor 3 and encoded by the SORT1 gene is a multiligand-binding single transmembrane spanning protein of the Vps10p family of sorting receptors. Similar to the (P)RR, sortilin-1 can act as a receptor for immature hormone peptides. In neurons, sortilin-1 regulates of cell faith signaling by pro-neurotrophic factors and controls secretion of the pro-brain derived neurotrophic factor. In addition, sortilin-1 was recently identified as a risk factor for cardiovascular diseases (CVD) [31-34] and shown to regulate LDL metabolism [35]. To confirm the interaction of (P)RR and sortilin-1, we immunoprecipitated endogenous (P)RR from crude HEK293 cell lysates. Immunoblotting with sortilin-1 antibodies showed sortilin-1 was co-purified by rabbit anti-(P)RR antibody but not with rabbit IgG as control (Figure 2A). Similarly, the (P)RR protein was immunodetected in immunocomplexes precipitated with mouse anti-sortilin-1 antibodies but not with mouse IgG (Figure 2B). These result confirms the association of the (P)RR with sortilin-1 in HEK293 cells as found in TAP purifications.

(P)RR controls sortilin-1 protein stability.

We next determined whether the interaction between the (P)RR and sortilin-1 is functionally relevant. For this, we suppressed (P)RR expression by transfecting HEK293 cells with small interfering RNA (siRNA) against the human (P) RR and immunoblotted whole cell lysates for (P)RR and sortilin-1 . (P)RR protein was decreased by 90% in cells transfected with (P)RR specific siRNA. Surprisingly, sortilin-1 protein was decreased by 50% in these cells, whereas control siRNA had no effect (Figure 4 A&B). Expression of LRP6, which is known to interact with the (P)RR [22], was not affected by (P)RR-depletions. Similarly, transfecting cells with sortilin-1 specific siRNA decreased sortilin-1 protein by more than 90%, and (P) RR protein by more than 50% (Figure 4 A&B). To determine whether the mutual regulation of protein abundance was caused by decreased mRNA levels, we measured (P) RR and sortilin-1 mRNA in siRNA-transfected cells by quantitative PCR. This showed that siRNAs against the (P)RR and sortilin-1 successfully suppressed target mRNA levels (Figure 5 A&B), but did not affect the mRNA levels of the other gene. This suggests that the decreased sortilin-1 expression in (P)RR knock-down cells and vice verse is not caused by changes in mRNA levels but decreased translation or protein stability.

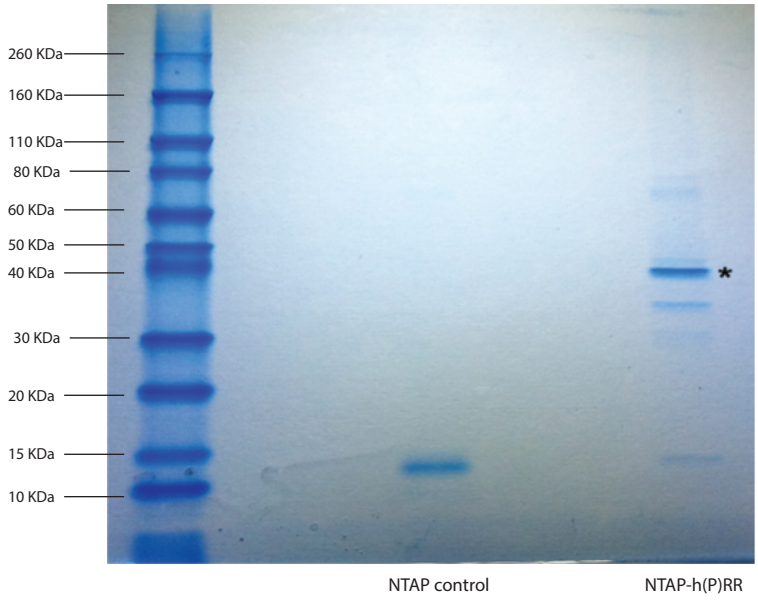

Figure 2. ***Purification of NTAP-h(P)RR.*** Final eluates from NTAP-control and NTAP-h(P)RR were separated on a 4-20% SDS-PAGE gel and visualized by colloidal coomassie staining The asterisk indicates the expected band for TEV-cleaved NTAP-h(P)RR.

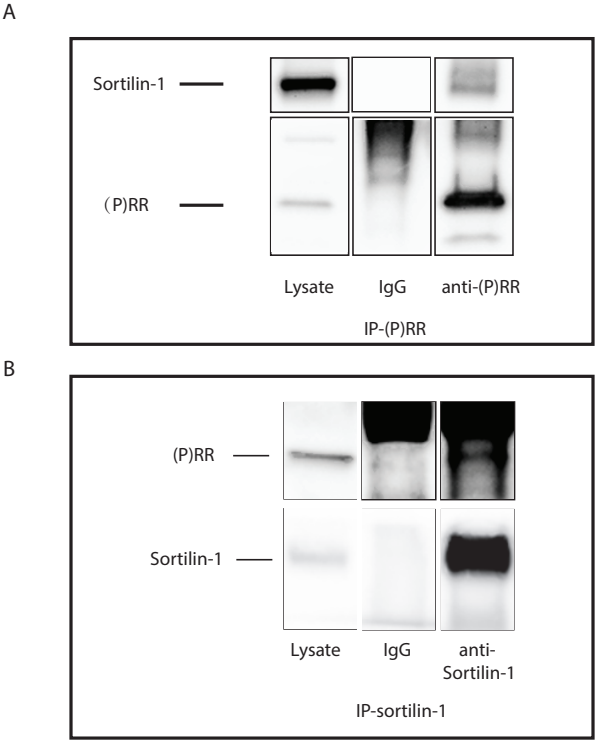

Figure 3. ***Interaction of endogenous (P)RR and sortilin-1.*** Co-precipitation of the (P)RR and sortilin-1, as indicated by immunoblotting whole cell lysates and immunocomplexes from HEK293 cells. Whole cell lysates (left panels), and lysates immunoprecipitated with (P)RR (A) and sortilin-1 (B) antibodies (right panels), or rabbit IgGs as control (middle panels) were immunoblotted for (P)RR or sortilin-1. The intense aspecific band in (P)RR immunoblots of rabbit IgG and sortilin-1 immunocomplexes are due to rabbit immunoglobulin heavy chains that run around 50 kD versus 38 kD for the (P)RR.

Discussion

We report the signature of a (P)RR-interactome from HEK293 cells. Two independent TAP-purifications revealed several proteins that specifically co-purified with NTAP-(P)RR, but not the NTAP-tag alone. As prove of concept, our profile contained V-ATPase subunits that have previously been shown to interact with the (P)RR [22]. Further analysis with one protein from our profile confirmed the association and colocalization of sortilin-1 with the (P)RR in HEK293 cells. Furthermore, by showing that the (P)RR and sortilin-1 mutually regulate protein abundance, we demonstrate that the interaction is of functional significance.

The (P)RR relays signals from different hormones to multiple downstream targets [36]. The mechanisms by which the (P)RR acts are as yet obscure, also due to the fact that the (P)RR has no homology to know receptors. A number of studies have indicated the importance of protein-protein interactions. (Pro)renin-binding to the (P)RR recruits the zinc finger protein PLZF to regulate gene expression [5, 11], whereas in Wnt-signaling, the (P)RR is required as an adaptor between the Fz/LRP receptor complex and the V-ATPase to regulate downstream signaling [22]. A recent study by Zaade et al. [37], however only showed a limited subset genes that are affected by (P)RR and PLZF depletion or (P)RR depletion and V-ATPase inhibition, which renders additional signaling mechanisms and binding partners likely. This is further underscored by the neurological phenotype of patients that are heterozygous for a truncated (P)RR and the inhibitory affect of this mutation on NGF signaling [25, 26].

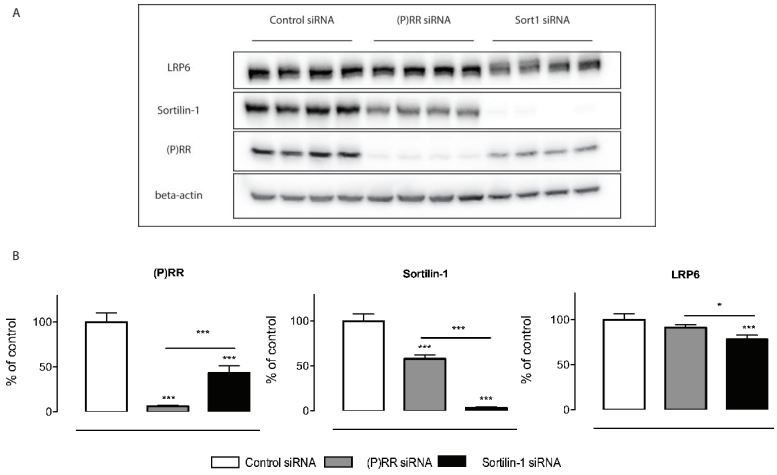

Figure 4. ***(P)RR and sortilin-1 mutually regulate protein abundance.*** siRNA knockdown of (P)RR expression decreased sortilin-1 protein abundance and vice verse. A: Representative western blots of lysates from control siRNA, (P)RR siRNA, and sortilin-1 siRNA transfected HEK293 cells. Samples from two independent experiments in duplicate were loaded for blotting. B: (P)RR, sortilin-1, and LRP6 protein levels, corrected for β-actin, were expressed as percentage of the siRNA control. *:P<0.05; ***:p<0.001; n=8 from 4 cell preparations.

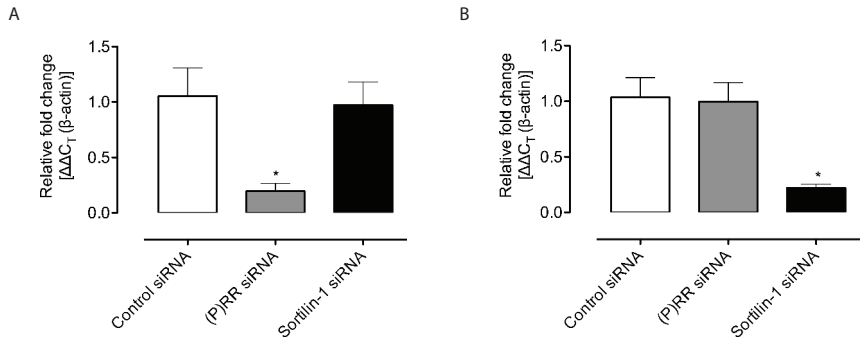

Figure 5. ***Reciprocal regulation of (P)RR and sortilin-1 protein abundance is not regulated at the transcriptional level.*** siRNAs against the (P)RR and sortilin-1 efficiently decreased target mRNA levels but did not affect the expression of the other gene. (P)RR (A) and sortilin-1 (B) mRNA levels in control siRNA, (P)RR siRNA or sortilin-1 siRNA transfected HEK293 cells, corrected for β-actin and expressed as fold change compared to control siRNA n=6 from 3 independent preparation; *:p<0.05.

We therefore used a hypothesis-free approach to find additional binding partners and putative novel functions, by purifying the (P)RR from HEK293 cells. We also eliminated bias for abundantly or stably binding proteins by not only selecting Colloidal Coomassie stained bands, but analyzing the entire SDS-PAGE lanes by LC/MS-MS. Our method was vindicated by the presence of the V0c and V0d subunits of the V-ATPase that have previously been shown to interact with the (P)RR, although the latter was only found in one purification. There were, however, also proteins previously identified as binding partners that were absent from the profile, for example PLZF and LRP6, even though HEK293 cells expressed LRP6 (Figure 4). There may be several reasons for these missing interactions. First, the TAP-tag may interfere with proper targeting or interactions with certain proteins. Second, the (P)RR can be cleaved by either furin or ADAM proteases [38, 39] in the extracellular domain close to the transmembrane to yield a soluble (P)RR (s(P)RR)) and a short transmembrane form, denoted as M8-9 that was originally found to be associated with the V-ATPase in chromaffin granules [14]. Since we fused the TAP-tag to the N-terminal domain of the (P)RR, any proteins that only interact with the M8-9 fragment would be lost from our profile. However, because of the deep conservation and predicted small size (24 amino acids) of the carboxyterminal intracellular domain [40], we preferred to tag the N-terminal domain. Third, we purified (P)RR-NTAP from serum grown HEK293 cells, whereas some interactions may be cell-type specific and depend on ligand (i.e. (pro) renin or Wnt) stimulation. Finally, some interactions may be lost during the purification procedure. Another limitation of our study was the presence of a number of hits that were recently reported to be commonly found as contaminants in affinity purification mass spectrometry data, the so called CRAPome [30], even though these proteins were not found in control purifications, and includes proteins involved in cytoskeletal organization, mitochondrial transport, DNA binding, translation and protein folding. Finally, we used stringent selection criteria by only listing proteins that were found in two independent

purifications. However, Vod and two V-ATPase associated proteins (below) were only found in one purification, but based on the (P)RR-V-ATPase association are likely true hits and therefore included. This renders the possibility of some of the other proteins that were only found in a single purification to be bona fide (P)RR-associated proteins likely.

Our profile did not provide hits that are obviously linked to (P)RR-dependent signaling, such as receptors or kinases. Nevertheless, we found a number of proteins that specifically interact with NTAP-(P)RR and are not recognized as common contaminants. Among these were two additional co-factors of the V-ATPase, ATP6AP1/Ac45 and VMA21, although both were only found in a single purification. Like the (P)RR, Ac45 is an accessory protein of the V-ATPase, harbors a single transmembrane domain and a large extracellular domain and can be proteolytically cleaved [41, 42]. Ac45 interacts with the V0-domain [43] and is required for lysosomal trafficking, granular acidification, and regulated exocytosis in osteoclasts, and neuroendocrine chromaffin and pituitary cells [44-46]. The VMA21 homologue in yeast, Vma21p, also interacts with the V0-domain and is indispensable for V0-assembly and insertion into the endoplasmic reticulum (ER), but not for the interaction between the V0- and V1-domains [47]. The finding of only V0-subunits or V0-domain associated accessory proteins suggests that the (P)RR is also specifically associated with the V0-domain. Furthermore, the presence of VMA21 raises the intriguing possibility that the association of VMA21 and the (P)RR is required for proper folding and insertion of the V0-domain in the ER. (P)RR depletion would then impair V0-domain assembly, resulting in increased degradation of free V0-subunits, which would explain the progressive disappearance of V0-subunits after (P)RR depletion [17-19, 21].

The most intriguing protein in our profile was sortilin-1, which was found in both purifications with high confidence. Like the (P)RR, sortilin-1 is a single transmembrane protein that can bind propeptide hormones, is involved in neuronal signaling and has been linked to cardiovascular disease (CVD). Sortilin-1, also known as neurotensin receptor 3 and encoded by the SORT1 gene is a multiligand receptor of the Vps10p family of sorting proteins that has diverse functions, for example in neurotrophic signaling and lipoprotein metabolism. In neurons, sortilin-1 controls secretion of the pro-brain
derived neurotrophic factor (proBDNF) [48], and regulates cell faith signaling by binding pro-neurotrophins [49] and targetting neurotrophin-receptors [50], which may explain why expression of dominant negative (P)RR inhibits NGF-signaling [26]. Recent genome-wide association studies (GWAS) revealed that the SORT1 locus is strongly associated with plasma low-density lipoprotein cholesterol (LDL) levels and the risk for CVD [51-53]. Subsequent studies have shown that sortilin-1 is a LDL clearing receptor that binds and internalizes LDL, and suppresses Apolipoprotein B (ApoB) secretion from the hepatic tissue, thereby controlling overall LDL homeostasis [54, 55], although conflicting data were found in Sort1-/- mice [35]. Furthermore, in the brain, sortilin-1 is an important clearance receptor for the complex of ApoE and the neurotoxic amyloid-β (Aβ) peptide that may indicate a role in Alzheimer's disease [56]. The

association between endogenous (P)RR and sortilin-1 was confirmed by mutual co-immunoprecipitations (Figure 3). The association between the (P)RR and sortilin-1 may have functional relevance, since knocking down (P)RR expression decreased the abundance of sortilin-1 protein, and vice versa. This is not due to transcriptional regulation, since mRNA levels were not affected (Figure 5), and would instead argue for changes in protein stability and turnover. How does this happen is not yet clear. It is known that low pH is required to trigger ligand-receptor dissociation that is necessary for receptor recycling, and the V-ATPase plays an important role in this cellular process [57]. As an example, recycling of PTH/PTHrP receptors is controlled by V-ATPase activity. Inhibiting V-ATPase activity by Bafilomycin A1 greatly reduced PTH/PTHrP receptor recycling in
LLCPK-1 cells [58]. It is thus possible that by coupling to the V-ATPase, (P)RR increases sortilin-1 recycling and prevents it from being targeted for lysosomal degradation. Sortilin-1 plays important role in protein sorting and trafficking. It controls intracellular sorting of BDNF, and depletion of sortilin-1 leads to a shift of BDNF from regulated secretory pathway to consecutive secretory pathway [59]. In addition, sortilin-1 has also been demonstrated to play important role in amyloid precursor protein lysosomal degradation, and alpha-1 antitrypsin traffkicking [60, 61]. Therefore, it is possible that sortilin-1 is also required for proper (P)RR targeting and prevents its targeting to lysosomes for degradation.

In summary, tandem-affinity purification of the (P)RR from HEK293 cells revealed a novel and functional interaction with sortilin-1. In light of the multiple functions of sortilin-1 in neuronal signaling, lipid metabolism and ApoE/Aβ-peptide clearance, this interaction may have important implications for brain development and several diseases, such as hypercholesterolemia and Alzheimer's disease.

Table 1. Mass Spectrometry analysis of (P)RR interacting proteins.

Symbol	Description	Accession	Exp 1 Unique peptide	Exp 1 Mascot score	Exp 2 Unique peptide	Exp 2 Mascot score	CRAPome freq (%)
ATP coupled cation transport							
ATP6AP2 *	(Pro)renin receptor	O75787	14	1136	23	1711	0
ATP1A1	Sodium/potassium-transporting ATPase subunit alpha-1	P05023	5	187	24	1290	20
ATP6V0C	V-type proton ATPase 16 kDa proteolipid subunit, V0 subunit c	P27449	1	62	1	86	1
ATP5A1	ATP synthase subunit alpha, mitochondrial	P25705	1	40	5	210	43
ATP6V0D1	ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d1	F5GYQ1	1	88	-	-	1
ATP6AP1	V-type proton ATPase subunit S1/AC45	Q15904	-	-	2	114	0
VMA21	Vacuolar ATPase assembly integral membrane protein VMA21	Q3ZAQ7	-	-	3	148	0
Mitochondrial transport							
SLC25A6	ADP/ATP translocase 3	P12236	14	829	16	1106	41
SLC25A5	ADP/ATP translocase 2	P05141	13	758	17	1183	47
SLC25A13	Calcium-binding mitochondrial carrier protein Aralar2	Q9UJS0	6	290	23	1389	11
SLC25A3	Phosphate carrier protein, mitochondrial	Q00325	5	233	12	654	23
SLC25A10	Mitochondrial dicarboxylate carrier	Q9UBX3	5	212	11	581	18
SLC25A1	Tricarboxylate transport protein, mitochondrial	P53007	4	176	7	349	9
SLC25A11	Mitochondrial 2-oxoglutarate/malate carrier protein	Q02978	3	136	9	561	8
SPNS1	Protein spinster homolog 1	Q9H2V7	2	72	4	142	1
SFXN4	Sideroflexin-4	Q6P4A7	1	40	2	69	0
Plasma membrane transport							
SLC16A1	Monocarboxylate transporter 1	P53985	4	189	6	340	5
SLC1A5	Neutral amino acid transporter B(0)	Q15758	2	66	6	321	8
Protein transport (Golgi)							
SEC61A1	Protein transport protein Sec61 subunit alpha isoform 1	P61619	2	68	5	240	4
Lipid metabolism							
CDIPT	CDP-diacylglycerol--inositol 3-phosphatidyltransferase	O14735	2	118	5	361	1
TECR	Trans-2,3-enoyl-CoA reductase	Q9NZ01	2	97	7	377	10

To be continued...

Table 1. Mass Spectrometry analysis of (P)RR interacting proteins (Continued).

Symbol	Description	Accession	Exp 1 Unique peptide	Exp 1 Mascot score	Exp 2 Unique peptide	Exp 2 Mascot score	CRAPome freq (%)
Lipid metabolism							
CDIPT	CDP-diacylglycerol--inositol 3-phosphatidyltransferase	O14735	2	118	5	361	1
TECR	Trans-2,3-enoyl-CoA reductase	Q9NZ01	2	97	7	377	10
Carbohydrate metabolism							
PHGDH	D-3-phosphoglycerate dehydrogenase	O43175	3	124	6	328	29
Protein folding and modification							
STT3A	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3A	P46977	5	197	7	272	3
UBA52	Ubiquitin-60S ribosomal protein L40	P62987	4	192	5	327	59
RPS27A	Ubiquitin-40S ribosomal protein S27a	P62979	4	192	5	327	62
UBC	Ubiquitin C	F5H2Z3	4	190	5	322	58
UBB	Polyubiquitin-B	P0CG47	4	180	5	311	58
DNAJA1	DnaJ homolog subfamily A member 1	P31689	4	170	11	674	23
DNAJA2	DnaJ homolog subfamily A member 2	O60884	3	127	7	388	17
DNA binding							
PRKDC	DNA-dependent protein kinase catalytic subunit	P78527	6	229	28	1170	33
RUVBL2	RuvB-like 2	Q9Y230	3	113	17	924	26
RUVBL1	RuvB-like 1	Q9Y265	2	80	13	648	25
Signal transduction							
SORT1	Sortilin-1	Q99523	8	462	15	856	0
ATAD3A	ATPase family, AAA domain containing 3A	Q9NVI7	7	305	8	379	15
PTPLAD1	3-hydroxyacyl-CoA dehydratase 3	Q9P035	1	56	2	125	8
TRAF4	TNF receptor-associated factor 4	B4DUU9	1	46	1	42	2
EMD	Emerin	P50402	1	43	6	262	13
Cytoskeletal organization							
TUBB4B	Tubulin beta-4B chain	P68371	13	760	22	1615	90
TUBA1B	Tubulin alpha-1B chain	P68363	10	546	19	1266	94
TUBB6	Tubulin beta-6 chain	Q9BUF5	7	349	12	794	73

To be continued

Table 1. Mass Spectrometry analysis of (P)RR interacting proteins (Continued).

				Exp 1		Exp 2		CRAPome
Symbol		Description	Accession	Unique peptide	Mascot score	Unique peptide	Mascot score	freq (%)
Unknown function								
	TMEM33	Transmembrane protein 33	P57088	2	87	3	200	5
	TMEM165	Transmembrane protein 165	Q9HC07	1	52	3	183	1

*: Bait

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Chapter 6

New Renin Inhibitor VTP-27999 Alters Renin Immunoreactivity and Does Not Unfold Prorenin

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Abstract

Renin inhibitors like aliskiren not only block renin but also bind prorenin, thereby inducing a conformational change (like the change induced by acid) allowing its recognition in a renin-specific assay. Consequently, aliskiren can be used to measure prorenin. VTP-27999 is a new renin inhibitor with an aliskiren-like IC50 and t1/2, and a much higher bioavailability. This study addressed (pro)renin changes during treatment of volunteers with VTP-27999 or aliskiren. Both drugs increased renin immunoreactivity. Treatment of plasma samples from aliskiren-treated subjects with excess aliskiren yielded higher renin immunoreactivity levels, confirming the presence of prorenin. Unexpectedly, this approach did not work in VTP-27999–treated subjects, although an assay detecting the prosegment revealed that their blood still contained prorenin. Subsequent in vitro analysis showed that VTP-27999 increased renin immunoreactivity for a given amount of renin by ≥30% but did not unfold prorenin. Yet, it did bind to acid-activated, intact prorenin and then again increased immunoreactivity in a renin assay. However, no such increase in immunoreactivity was seen when measuring acid-activated prorenin bound to VTP-27999 with a prosegment-directed assay. The VTP-27999–induced rises in renin immunoreactivity could be competitively prevented by aliskiren, and antibody displacement studies revealed a higher affinity of the active site-directed antibodies in the presence of VTP-27999. In conclusion, VTP-27999 increases renin immunoreactivity in renin immunoassays because it affects the affinity of the active site-directed antibody. Combined with its lack of effect on prorenin, these data show that VTP-27999 differs from aliskiren. The clinical relevance of these results needs to be established.

Introduction

Renin belongs to the A1 family of aspartic proteases. Its 3-dimensional (3D) structure consists of 2 β-sheet domains (N and C domain) related by an ≈2-fold axis. The active site is a deep cleft between the N and C domains that extends over 8 residues of renin's substrate, angiotensinogen. Each domain supplies 1 catalytic aspartic acid residue in the center of the binding cleft. A long β-hairpin loop structure called the flap in the N-terminal domain covers the central part of the angiotensinogen-binding site, and the conformation with the tip of the flap open allows the entrance of the substrate and removal of hydrolytic products during the catalytic turnover, while the tip closes on substrate binding.1 In the case of renin's inactive precursor, prorenin, the flap is open to accommodate the 43-amino acid prosegment [1,2]. Morales et al recently proposed that in prorenin [2], the catalytic binding site is covered by the N-terminal part of mature renin that, in turn, is covered by the prosegment. After removal of the prosegment, this N-terminal part becomes part of a 6-stranded β-sheet on the back of the mature renin molecule, previously occupied by the prosegment. This requires a conformational change that fully exposes the active cleft [2].

Prosegment unfolding occurs in a pH- and temperature-dependent manner and, if not followed by cleavage, results in 2 prorenin conformations as follows: a closed, inactive form, and an open form that displays full enzymatic activity (Figure 1) [3,4]. In addition, an intermediate form exists where the prosegment has moved away from the cleft, but where the renin part still has to undergo the above-mentioned conformational changes. Under physiological conditions, <2% of prorenin is in the open conformation. The recently introduced renin inhibitor, aliskiren (Figure S1A in the online-only Data Supplement), binds to prorenin in the open conformation, as well as to the intermediate form of prorenin [3,4]. Binding to the intermediate form induces prorenin unfolding. Because of the tight binding of the renin inhibitor, the refolding step (ie, the return to the closed conformation) is no longer possible, and thus the equilibrium between the closed and open conformation will shift in favor of the open conformation. Eventually, depending on the concentration of aliskiren, a significant proportion of prorenin may be open (nonproteolytic activation), allowing its recognition by the active site-directed antibodies used in renin immunoradiometric assays (IRMAs), despite the fact that the prosegment is still present and aliskiren is bound to the active site [5,6]. Because prorenin levels are, on average, ≈10-fold higher than those of renin, this may affect immunoreactive renin measurements in patients taking aliskiren [7,8]. A direct prorenin ELISA, based on the recognition of an epitope near prorenin's putative cleavage site, helps to circumvent this problem [4]. This assay also detects the intermediate form of prorenin but not the open, active form.

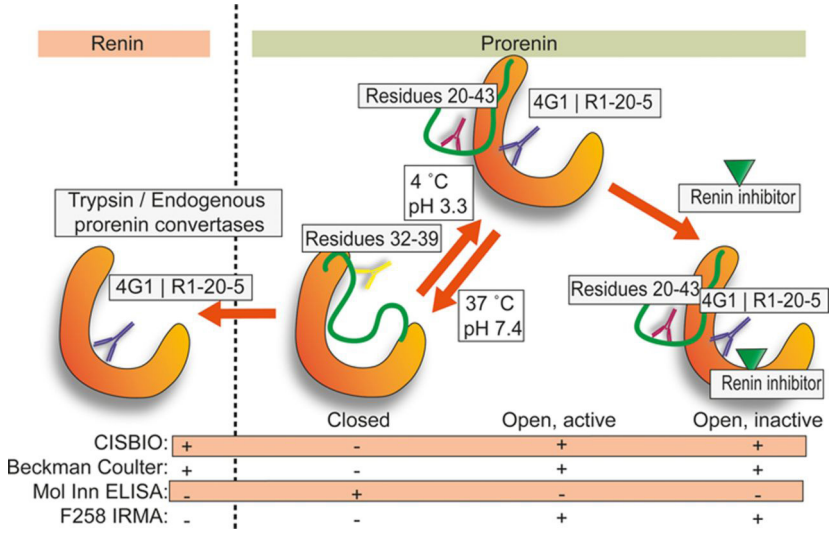

Figure 1. Conformational changes and the expression of immunoreactive epitopes associated with the activation of prorenin. Shown are closed, intact prorenin, open, intact prorenin (active, or, following renin inhibitor binding, inactive), and trypsin-cleaved prorenin (renin), as well as the various assays that recognize these forms. Please note that low pH and low temperature will cause prorenin unfolding, and that a renin inhibitor locks prorenin in this open conformation, even when the pH and temperature return to normal. IRMA indicates immunoradiometric assay. CISBIO and Beckman Coulter refer to the respective renin IRMAs.

VTP-27999 (Figure S1B) is a new, active site-directed renin inhibitor with an IC50 (0.3 nmol/L) and half life (30 hours) that are comparable to those of aliskiren (0.6 nmol/L and 40 hours, respectively), but with a much higher bioavailability (>20% versus 2.6%) [9]. This study tested the changes in renin and prorenin during a 10-day treatment of salt-depleted volunteers with 150 mg VTP-27999, focusing on the possibility that the VTP-27999-induced rise in renin is attributable, at least in part, to prorenin unfolding. Remarkably, this turned out not to be the case. Therefore we also performed an extensive biochemical analysis of the effects of VTP-27999 on renin and prorenin in vitro. A direct comparison with aliskiren revealed that both drugs have a different mode of action. To what degree this has clinical relevance remains to be determined.

Methods

Studies in Humans

Healthy volunteers (9 men, 1 woman; age 19–41 years) received a low-salt (10 mEq/d) diet for 5 days and were then given 150 mg VTP-27999 (n=6), 300 mg aliskiren (n=2), or placebo (n=2) once daily for 10 days, followed by a washout period of 72 hours, while continuously maintaining the low-salt diet. EDTA plasma was collected at various time points on day 1, 2, 10, 11, 12, and 13. Written, informed consent was obtained from each volunteer, and the protocol was approved by the PRACS Institute, Ltd, Institutional Review Board, Fargo, ND.

Studies in Plasma and Buffer

To study whether VTP-27999 affects the measurement of renin or prorenin, recombinant human renin and prorenin (0.19 and 0.10 mg/mL, respectively, a gift from Actelion Pharmaceuticals, Alschwill, Switzerland) were diluted in human plasma or phosphate buffer (0.0122 mol/L $NaH_2PO_4 \bullet 2H_2O$, 0.0867 mol/L Na_2HPO_4, 0.0759 mol/L NaCl; pH 7.4) containing 0.1% BSA (Sigma) and incubated at 4, 22, or 37°C in the absence or presence of VTP-27999 (at concentrations ranging from 0.001 to 100 µmol/L) for various time periods, after which renin, open prorenin, and total prorenin were measured. For comparison, similar studies were performed with aliskiren, either alone or on top of VTP-27999, and in plasma containing endogenous renin and prorenin, both before and after activating prorenin with trypsin.

Next, to address whether VTP-27999 binds to open, intact prorenin, recombinant human prorenin was fully unfolded (without cleaving off the prosegment) by incubating it in an acidic solution (0.05 mol/L glycine, 0.051 mol/L EDTA, 0.0949 mol/L NaCl; 0.1% BSA; pH 3.3) for 48 hours at 4°C.10 Thereafter, the samples were neutralized with ice-cold phosphate buffer containing 0.1% BSA in the absence or presence of VTP-27999 or aliskiren (at concentrations ranging 1–10 µmol/L), and incubation continued at 37°C for up to 1 hour, after which the assays discussed below were performed immediately at 4°C.

Finally, to investigate the possibility that VTP-27999 binding to renin affects the affinity of the monoclonal antibody recognizing renin's active site, renin was determined at a fixed concentration (≈200 pg/mL) by renin IRMA with or without 0.1 μmol/L VTP-27999 in the presence of increasing concentrations (1 pmol/L to 1μmol/L) of the nonlabeled active site-directed antibody R1-20-5.

Biochemical Assays

Renin was measured with the renin III (Cisbio, France) IRMA (detection limit 1 pg/mL). This assay, which makes use of a monoclonal antibody (4G1) [11] directed against renin's active site, also recognizes intact, open prorenin [3,12] This implies that intact prorenin can be measured with this assay after incubating it with acid or after exposing it, for 48 hours at 4°C, to 10 μmol/L aliskiren because both procedures induce the conversion of all prorenin molecules into the open conformation [5]. Additionally, we converted prorenin to renin by cleaving off the prosegment with immobilized trypsin (72 hours at 4°C). In plasma, this approach yields identical total renin (renin+prorenin) levels as aliskiren exposure and thus, subtracting the renin levels measured before trypsin treatment or aliskiren exposure from those after these procedures indirectly provides an indication of the prorenin levels. (Figure 1)

Intact, closed prorenin was measured with an ELISA (detection limit 10 pg/mL) that recognizes residues 32 to 39 of the prosegment (Molecular Innovations, Novi, MI) [4]. This prorenin assay was performed according to the instructions of the manufacturer, making use of the above-mentioned human recombinant prorenin to construct the standard curve. In a select set of samples, intact, open prorenin was measured on the basis of its prosegment, replacing the 125I-labeled active site-directed monoclonal antibody of the Cisbio kit by a prosegment-directed 125I-labeled monoclonal antibody (F258-37-B1) in the IRMA (F258 IRMA; detection limit 10 pg/mL). F258-37-B1 is directed against the C-terminal part (p20–p43) of the propeptide and does not react (<0.1%) with renin. F258-37-B1 also does not react (<0.1%) with intact, closed prorenin [13]. However, it does react with prorenin after the above treatment of prorenin with aliskiren. Thus, the aliskiren-induced nonproteolytic conformational change, causing the propeptide to move to the surface of the molecule, allows the recognition of prorenin by both the active site-directed antibody of the Cisbio kit and the prosegment-directed antibody of the prorenin IRMA.

Finally, because VTP-27999 seemed to affect the outcome of the Cisbio IRMA, renin measurements in the presence of VTP-27999 were also performed with an alternative renin IRMA (Beckman Coulter, Immunotech, Prague, Czech Republic). This IRMA makes use of the active site-directed monoclonal antibody R1-20-5 [10], and has a detection limit of 0.9 pg/mL.

Data Analysis

Results are shown as mean±SEM. Differences were tested using 1-way ANOVA, followed by Dunnett multiple comparison test; $P<0.05$ was considered significant.

Results

Measurements in Plasma of Placebo-, Aliskiren-, or VTP-27999–Treated Subjects

In placebo-treated subjects, plasma renin levels remained stable throughout the entire treatment period (Figure 2A). In vitro incubation of plasma with 10 µmol/L aliskiren resulted in a substantial rise in the immunoreactive renin levels detected by the renin IRMA. This is attributable to the fact that the assay now also recognizes prorenin (converted to an open, renin-like conformation by aliskiren; ie, it determines total renin). The difference between total renin (measured after aliskiren exposure) and renin (measured before aliskiren exposure) reflects the prorenin levels. Prorenin, like renin, remained stable in the placebo-treated subjects during the entire treatment period.

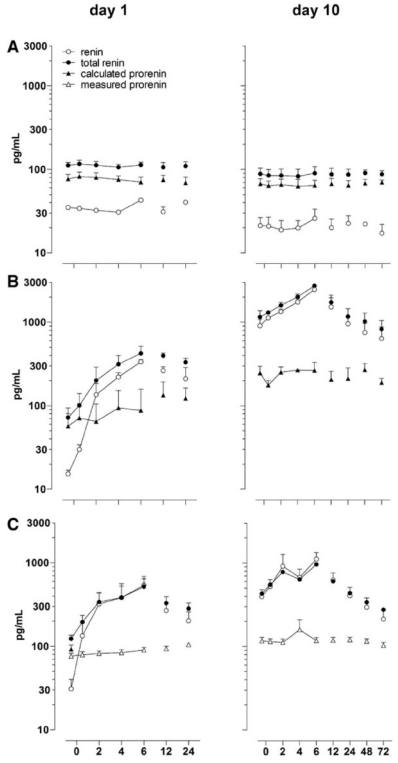

Figure 2. Renin, total renin, and prorenin concentrations in plasma of healthy volunteers receiving placebo (A), 300 mg aliskiren (B), or 150 mg VTP-27999 (C) during 10 days, followed by a 3-day washout period. Prorenin was either calculated by subtracting renin from total renin or measured directly. Data are mean±SEM of n=2 to 6.

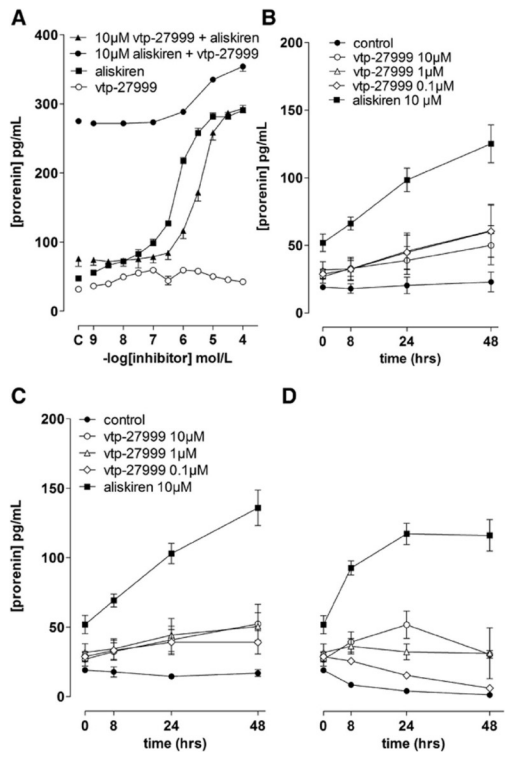

Figure 3. A, Prorenin measured by renin immunoradiometric assay (IRMA) after its incubation for 48 hours at 4°C in buffer with aliskiren alone, VTP-27999 alone, aliskiren after a 24-hour preincubation with 10 µmol/L VTP-27999, or VTP-27999 after a 48-hour preincubation with 10 µmol/L aliskiren. B–D, Prorenin measured by renin IRMA after its incubation in plasma for 48 hours at 4°C (B), 22°C (C), or 37°C (D) in the absence (control) or presence of VTP-27999 or aliskiren at various concentrations. Data are mean±SEM of n=3 to 4.

Aliskiren treatment acutely increased renin >20-fold on day 1, and further increases (up to 100-fold) were observed on day 10 (Figure 2B). Renin levels decreased with a half life of 39±10 hours after stopping treatment on day 10, in agreement with the half life of aliskiren. In vitro exposure to 10 μmol/L aliskiren yielded the total renin levels, and from these levels, prorenin could be calculated. As expected on the basis of its constitutive release, prorenin levels started to rise only after >12 hours and rose further during prolonged treatment. Prorenin levels had not yet returned to baseline before the end of the protocol (on day 13).

VTP-27999, like aliskiren, greatly increased renin levels on day 1, and further increases were observed on day 10 (Figure 2C). Renin levels decreased with a half life of 30±3 hours after stopping treatment, in agreement with the half life of VTP-27999. Unexpectedly, in vitro exposure to aliskiren of plasma samples, obtained from VTP-27999-treated subjects at ≥2 hours after VTP-27999 intake, did not yield total renin levels that were higher than the renin levels measured before aliskiren exposure. In some cases, the levels were even lower. On average, the levels were identical with and without aliskiren exposure. This was not the case in the samples taken during the first 2 hours after VTP-27999 intake nor in the samples taken after stopping treatment; in those samples aliskiren did increase the amount of immunoreactivity in the renin IRMA.

The lack of an aliskiren-induced rise in immunoreactive renin levels in samples from VTP-27999-treated subjects suggests that either prorenin had disappeared during such treatment or that VTP-27999 had already converted all prorenin molecules into an open conformation in vivo, leaving no additional effect to be induced by aliskiren in vitro. To investigate this, we measured intact, closed prorenin with the direct prorenin ELISA (Figure 2C). This assay clearly demonstrated the presence of prorenin during VTP-27999 treatment, behaving identically as after aliskiren treatment: a modest rise after >12 hours and a further rise at day 10, with no return to baseline yet on day 13. Moreover, the prosegment-directed F258 assay (which recognizes prorenin only when the prosegment has moved out of the active cleft, ie, in its open form) yielded no detectable prorenin levels in plasma samples of either placebo-treated (n=2) or VTP-27999-treated (n=5) subjects (data not shown). This confirms that plasma prorenin in VTP-27999-treated subjects was present in its closed conformation.

Additionally, we measured total renin in a subset of samples (obtained on day 10 between 4 and 6 hours after dosing) after incubating plasma samples of VTP-27999-treated subjects with immobilized trypsin to cleave off the prosegment. In these samples, immunoreactive renin levels rose from 1587±493 before, to 1813±524 pg/mL (n=6; $P<0.005$) after trypsin exposure. From these levels, plasma prorenin levels of 225±46 pg/mL could be calculated. These levels are ≈2-fold higher than the prorenin levels measured with the direct ELISA in the same samples (109±9 pg/mL; $P<0.05$).

Taken together, these data show that intact prorenin is still present after VTP-27999 but that the (indirect) aliskiren approach underes-

timates its levels, whereas the trypsin approach overestimates its levels.

Studies With Recombinant Human Prorenin

Next, we verified the effect of aliskiren and VTP-27999 on prorenin in vitro. Incubating recombinant human prorenin with aliskiren at increasing concentrations at 4°C for 48 hours resulted in prorenin unfolding, allowing its recognition in the renin IRMA (Figure 3A; n=4; pEC50=6.2±0.02 [pEC50 indicates the negative logarithm of the inhibitor concentration at which 50% of the maximum effect has been reached]). In agreement with previous studies [5,6], 10 μmol/L aliskiren was sufficient to fully unfold all prorenin molecules. Remarkably, incubation with VTP-27999 did not result in prorenin unfolding (n=4). Yet, a 24-hour preincubation at 4°C with VTP-27999 shifted the aliskiren unfolding curve ≈10-fold to the right (n=4; pEC50=5.4±0.04; P<0.005 versus without VTP-27999). This illustrates that VTP-27999 and aliskiren display competition for the same binding site. When performing the reverse experiment (a 24-hour preincubation with aliskiren followed by a 48-hour incubation with VTP-27999), VTP-27999 unexpectedly increased the amount of immunoreactivity in the Cisbio assay by ≈30% in a concentration-dependent manner (n=4; pEC50=5.4±0.1).

To determine whether temperature or plasma components are important determinants of prorenin activation by VTP-27999, we also studied unfolding at 0.1, 1, and 10 μmol/L VTP-27999 in human plasma at 4, 22, and 37°C, using 10 μmol/L aliskiren as a positive control (n=3 for each condition). Yet, again, no effect of VTP-27999 was seen (Figure 3B and 3D). In agreement with this observation, incubation of prorenin for 48 hours at 4°C with VTP-27999 (1 nmol/L to 100 μmol/L) also did not allow its detection in the F258 assay (n=3, data not shown). Thus, both aliskiren and VTP-27999 bind to prorenin, but only aliskiren is capable of inducing prorenin unfolding.

Studies With Acid-Activated Prorenin

Subsequently, we activated (ie, unfolded) prorenin with acid.10 This procedure converts all prorenin molecules into the open, active conformation, allowing their detection in the renin IRMA (Figure 1). Neutralization will result in rapid refolding of prorenin so that it can no longer be detected. Thus, by measuring acid-activated prorenin in the renin IRMA at various time points after neutralization, one gets an indication of the velocity of the refolding process. At 37°C, open prorenin returned to its closed, inactive conformation with a t1/2 of 10±1 minutes (Figure 4A and 4B; n=4). This will not occur when locking prorenin in the open conformation, for example, because of the binding of a renin inhibitor to the active site (Figure 1). Indeed, both aliskiren and VTP-27999 prevented the refolding process in a concentration-dependent manner, and at concentrations of ≥100 nmol/L, both drugs fully locked prorenin in its open conformation (Figure 4A and 4B; n=3–4 for each concentration). Their affinities for open prorenin were identical (Figure 4C; pIC50 8.2±0.1 versus 8.4±0.1). These data indicate that VTP-27999 is capable of binding to prorenin's active site when it is exposed, and that once bound, it keeps prorenin in the open, unfolded conformation.

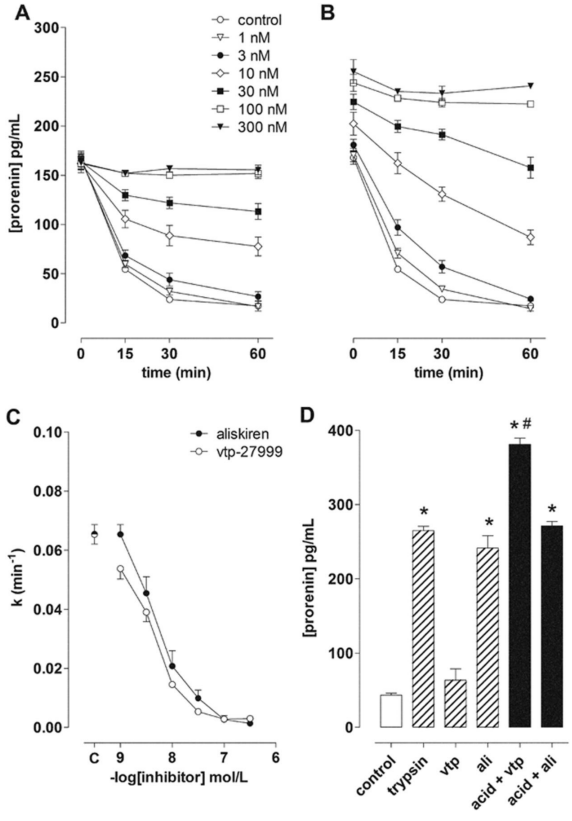

Figure 4. A and B, Time-dependent refolding (inactivation) of acid-activated recombinant human prorenin at 37°C in buffer in the absence (control) or presence of increasing concentrations of aliskiren (A) or VTP-27999 (B). C, First order rates of prorenin inactivation calculated from the data in A and B. D, Prorenin measured by renin immunoradiometric assay after its incubation with buffer (control), trypsin, 10 µmol/L VTP-27999, 10 µmol/L aliskiren, or acid combined with VTP-27999 or aliskiren. Data are mean±SEM of n=3 to 4. *P<0.05 vs control. #P<0.05 vs trypsin.

Unexpectedly, as demonstrated in Figure 4B, VTP-27999 not only blocked prorenin refolding but also increased the absolute levels detected in the renin IRMA by 41±7%. To investigate the latter in further detail, we applied the renin IRMA to a fixed concentration of prorenin, either without pretreatment, after incubation with trypsin, 10 µmol/L aliskiren or 10 µmol/L VTP-27999, or after acid pretreatment followed by neutralization in the presence of aliskiren or VTP-27999 (both 10 µmol/L). As expected, trypsin, aliskiren alone, and acid+aliskiren yielded the same maximum renin immunoreactivity level, and VTP-27999 alone did not unfold prorenin (Figure 4D; n=3 for each condition). Acid+VTP-27999 (n=3) again resulted in the detection of 40% more renin immunoreactivity, similar to the increases observed in Figures 3A and 4B. This suggests that VTP-27999 binding to the active site of unfolded prorenin either allows the detection of a prorenin variant that previously remained undetected (thus resulting in higher prorenin levels) or affected the binding affinity of the active site-directed antibody of the renin IRMA. To verify the former, we also measured acid-activated prorenin on the basis of its prosegment (with the F258 assay) in the presence of 100 nmol/L VTP-27999. This assay did not reveal an increase in immunoreactivity after VTP-27999 (98±4 versus 117±11 pg/mL in the presence of 10 µmol/L aliskiren; n=4), thereby excluding the concept that we detected a new prorenin variant with the renin IRMA that could previously not be detected.

In summary, both VTP-27999 and aliskiren lock unfolded prorenin in its open conformation, allowing its detection in a renin IRMA. VTP-27999 binding causes an additional rise in renin (but not prosegment) immunoreactivity, most likely because such binding selectively affects the affinity of the active site-directed antibody.

Studies With Renin

To verify whether VTP-27999 affected the detection of renin, we quantified recombinant human renin in phosphate buffer in the presence of increasing concentrations of VTP-27999. When present during the assay procedure, VTP-27999 concentration dependently increased the amount of renin detected in the renin IRMA (Figure 5A; n=3), until at VTP-27999 concentrations of ≥10 nmol/L, consistently 30% to 40% higher renin levels were detected, identical to the rise observed in Figure 4D for acid-activated prorenin. Such increases were not observed with aliskiren at concentrations up to 1 µmol/L. Importantly, when preincubating renin in buffer with VTP-27999 (0.1, 1, or 10 µmol/L; n=4 for each condition) for 60 minutes at room temperature and then performing the renin assay in the presence of increasing aliskiren concentrations, it became clear that aliskiren annihilates the effect of VTP-27999 in a concentration-dependent manner (IC50, 0.3±0.2, 1.8±0.5, and 21±7 µmol/L, respectively; Figure 5B).

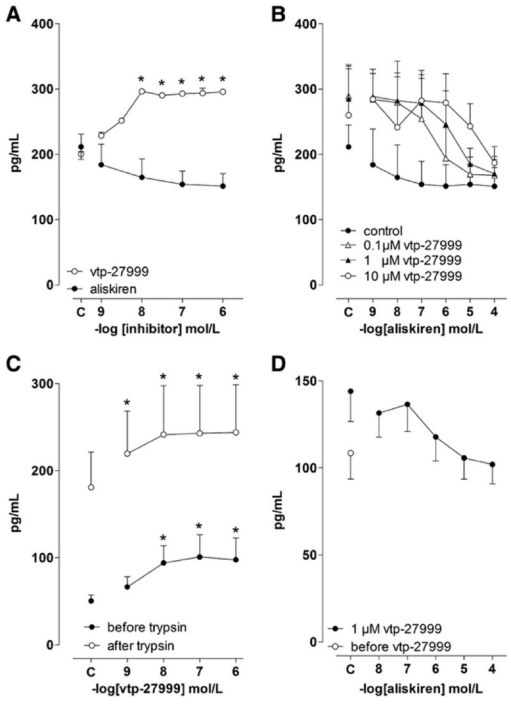

Figure 5. A, Recombinant human renin measured in buffer in the absence (control, C) or presence of increasing concentrations of VTP-27999 or aliskiren. B, Recombinant human renin measured in buffer in the absence (control, C) or presence of increasing concentrations of aliskiren after its preincubation with VTP-27999 (0.1–10 µmol/L) for 1 hour. C, Renin in human plasma measured before and after trypsin activation of prorenin in the absence or presence of increasing concentrations of VTP-27999. D, Renin in trypsin-pretreated human plasma measured in the absence (control, C) or presence of increasing concentrations of aliskiren after its exposure to VTP-27999. Data are mean±SEM of n=3 to 5. *P<0.05 vs control.

Given the above findings on recombinant human renin, we also wanted to know whether this applies to endogenous renin. To this end, plasma samples of nonrenin inhibitor-exposed subjects (n=5), before and after trypsin activation, were measured in

the presence of increasing concentrations of VTP-27999. Similar to recombinant human renin, maximum increases in renin immunoreactivity were observed at VTP-27999 concentrations of ≥10 nmol/L. Before trypsin, these increases amounted to maximally 30±3 pg/mL (+70±6%), whereas after trypsin, these increases amounted to 48±4 pg/mL (+35±1%; Figure 5C; P<0.05 versus before trypsin). The larger absolute rise after trypsin indicates that the increase in renin immunoreactivity involved both renin and trypsin-activated prorenin. Like in buffer, the VTP-27999 (1 µmol/L)-induced rise in renin immunoreactivity could be fully reversed by aliskiren (IC50 0.8±0.2 µmol/L; Figure 5D). Furthermore, aliskiren (0.01–100 µmol/L) decreased the level of renin immunoreactivity in 4 trypsin-pretreated plasma samples of VTP-27999–treated subjects, taken between 4 and 6 hours after oral dosing on day 10, by maximally 27±1% (data not shown).

In summary, VTP-27999 binding to renin increases renin immunoreactivity by ≈30%, and aliskiren can annihilate this effect in a competitive manner. Thus, both inhibitors compete for the same binding sites, but only VTP-27999 binding affects the affinity of the active site-directed antibody. Not surprisingly, this phenomenon also applies to trypsin-cleaved prorenin. These observations imply that the renin levels in plasma samples of VTP-27999-treated subjects may have been overestimated by ≈30%. Preincubation of such samples with high aliskiren levels (100 µmol/L) prevents this phenomenon.

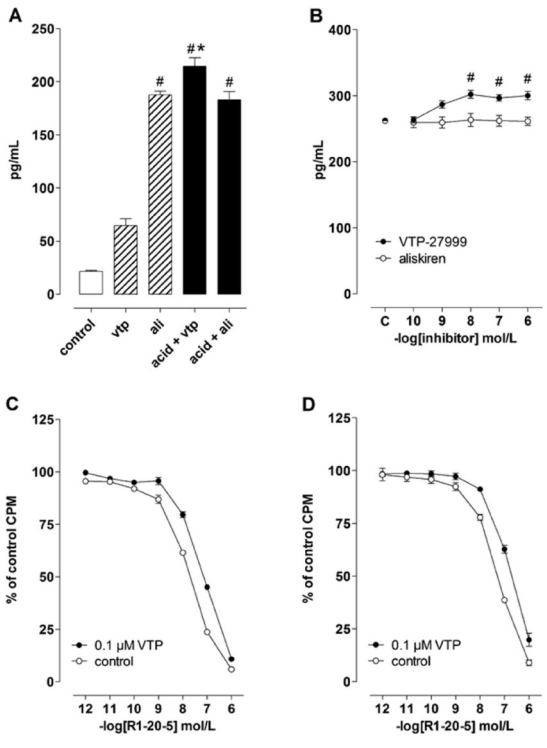

Figure 6. A, Recombinant human prorenin measured by Beckman Coulter renin immunoradiometric assay (IRMA) after its incubation with buffer (control), 10 µmol/L VTP-27999, 10 µmol/L aliskiren, or acid combined with VTP-27999 or aliskiren. B, Recombinant human renin measured in buffer by Beckman Coulter IRMA in the absence (control, C) or presence of increasing concentrations of VTP-27999 or aliskiren. C and D, Concentration-dependent prevention of the binding of 125I- labeled secondary antibody R1-20-5 (C, Beckman Coulter IRMA) or 4G1 (D, Cisbio IRMA) to renin (≈200 pg/mL, bound to the primary antibody) by unlabeled R1-20-5, in the absence and presence of 0.1 µmol/L VTP-27999. Results are expressed as a percentage of the counts per minute (CPM) in the control situation without the unlabeled antibody (7.759±251 vs 10.038±78 in the Beckman Coulter IRMA, and 17.040±219 vs 24.639±417 in the Cisbio IRMA in the absence and presence of VTP-27999, respectively). A rightward shift was observed in each individual experiment. #P<0.05 vs untreated control. *P<0.05 vs aliskiren. Data are mean±SEM of n=3.

Does VTP-27999 Alter the Affinity of the Active Site–Directed Antibodies?

Making use of a renin IRMA (Beckman Coulter) that applies a different active site-directed antibody (R1-20-5 versus 4G1 in the Cisbio IRMA; Figure 1), we were able to confirm the increase in renin immunoreactivity after VTP-27999 exposure, both in the case of acid-activated prorenin (Figure 6A; n=3) and renin (Figure 6B; n=2). Moreover, in the presence of VTP-27999, it took ≈4-fold higher R1-20-5 concentrations to prevent the binding of 125I-labeled R1-20-5 (Figure 6C; pIC50 7.7±0.01 versus 7.1±0.01; n=3) or 125I-labeled 4G1 (Figure 6D; pIC50 7.3±0.03 versus 6.7±0.01; n=3) than in the absence of VTP-27999. Thus, VTP-27999 binding to the active site increases the affinity of these 2 active site-directed antibodies. This explains the higher immunoreactivity levels of acid-activated prorenin and renin in the presence of VTP-27999 (Figures 4D, 5A, and 5C).

Discussion

This study shows that the renin inhibitor VTP-27999 acts differently from aliskiren. First, VTP-27999 binding to renin increases renin immunoreactivity in 2 different renin IRMAs. Second, in contrast to aliskiren (and to other renin inhibitors, including remikiren) [5,6], VTP-27999 does not induce prorenin unfolding. It does, however, bind to acid-activated, open prorenin, and by doing so, it keeps prorenin in this open conformation and then again increases the level of renin immunoreactivity when measuring this open prorenin in a renin IRMA.

Adding VTP-27999 at concentrations of ≥10 nmol/L during the renin IRMA of plasma samples increased the detected amount of immunoreactive renin by ≥30%. Similar increases were observed when measuring recombinant human renin in buffer. Aliskiren was capable of preventing the VTP-27999 effect in a concentration-dependent manner. This demonstrates that VTP-27999 and aliskiren compete for the same binding site, but that only VTP-27999 binding produces a surface that increases the affinity of the active site–directed antibody. Interestingly, on the basis of crystallization studies, Rahuel et al [14], have proposed the existence of 2 renin conformations: an open, uninhibited conformation, and a closed, inhibited formation. In the closed structure, the C-terminal loop segments forming the active site are closer to those from the N-terminal domain than in the open structure. Crystallographic analysis of the binding of aliskiren and VTP-27999 to these 2 forms reveals that the 2 inhibitors bind to both conformations, and that on binding, the flap is closed (Figures S2–S4). In the case of VTP-27999, the conformations of the 2 inhibited monomers are nearly identical, whereas with aliskiren, the 2 monomers differ from one another by the movement of the C-terminal domain closing down over the aliskiren-filled binding pocket. To what degree these conformational differences would alter the affinity of the active site-directed antibodies is unknown. Unfortunately, the binding epitopes of the 2 active site-directed monoclonal antibodies applied in our study (which were generated >30 years ago) are unknown. Nevertheless, our data show clearly that, in the presence of VTP-27999, more unlabeled antibody is required to displace the 125I-labeled active site-directed antibody, thereby

demonstrating that binding occurs with higher affinity in the presence of VTP-27999.

The VTP-27999–induced increase in renin immunoreactivity also occurred when measuring trypsin-activated prorenin and acid-activated prorenin. The former is not surprising because trypsin converts prorenin to renin. The latter is in agreement with the observation that acid-activated, unfolded prorenin has the same 3D conformation as renin (and displays full enzymatic activity) [10,15], although it still contains the prosegment. The presence of the prosegment allowed us to measure VTP-27999-bound, unfolded prorenin also with a second antibody directed against the prosegment (F258 IRMA, Figure 1). With this antibody, no increase in immunoreactivity was observed in the presence of VTP-27999. Therefore, it seems that VTP-27999 affected the binding of the active site-directed antibodies only.

The conversion of closed, inactive prorenin to open, active prorenin is a 2-step process. First, the prosegment moves out of the cleft, resulting in an intermediate form that cannot be detected in a renin IRMA and that does not display activity. Next, this intermediate form undergoes the required conformational changes making it indistinguishable from renin. Aliskiren and other renin inhibitors bind to the intermediate form and subsequently induce prorenin unfolding. Because of their tight (almost irreversible) binding, the equilibrium will shift into the direction of the open form [3,4]. To fully convert all prorenin molecules into the open form, aliskiren concentrations of 10 μmol/L are required [5]. Such high concentrations do not occur in vivo during aliskiren treatment [16]. Nevertheless, even if only a small percentage of prorenin obtains the open conformation in vivo during aliskiren treatment, this may already result in an overestimation of the renin rise during renin inhibition [4,7,8]. Our current data show that *ex vivo* exposure of plasma samples obtained from aliskiren-treated subjects to 10 μmol/L aliskiren still led to further rises in immunoreactive renin. Thus, indeed not all prorenin molecules had already obtained the open conformation in vivo during aliskiren treatment, in full agreement with previous studies [4,7,8]. Yet, *ex vivo* exposure of plasma samples obtained from VTP-27999–treated subjects to aliskiren did not result in a further rise in immunoreactive renin, and in some samples even a decrease occurred.

At first sight, this suggests that VTP-27999, in contrast to aliskiren, had already converted all prorenin molecules into the open conformation in vivo. However, we were unable to detect unfolded prorenin (with the F258 assay) in plasma samples of VTP-27999–treated subjects. Moreover, in vitro studies revealed that VTP-27999 is incapable of inducing prorenin unfolding.

An explanation of these findings can now be derived from our observations on immunoreactive renin after its incubation with VTP-27999. VTP-27999 acutely increases the results of the renin assay by ≥30%, and aliskiren (partially) blocks this effect. Thus, at renin levels that are >2 orders of magnitude above normal (and 10-fold higher than prorenin; Figure 2C), VTP-27999 will yield an increase in renin immunoreactivity that is far higher than the actual prorenin levels (eg, at a renin level of 1000 versus

100 for prorenin, the renin assay will detect 1300). Even when aliskiren fully displaces VTP-27999 from renin, thereby diminishing its increasing effect (ie, the renin level goes back from 1300 to 1000), this will not allow us to detect the simultaneously occurring (much smaller!) increase in immunoreactivity attributable to aliskiren-dependent prorenin activation (+100 in the above example, resulting in a total renin level of 1100 after aliskiren versus 1300 before aliskiren). Consequently, depending on the actual VTP-27999 levels (≈30 [trough]–1300 [peak] nmol/L; R. Gregg, unpublished data), the renin rise and the prorenin levels, both rises and decreases in renin immunoreactivity may be expected after aliskiren exposure in vitro. This is exactly what happened. Thus, in samples of VTP-27999–treated subjects, prorenin can only be detected indirectly using the trypsin method or directly by measuring prorenin with a direct prorenin assay. The results of the latter assay were lower than when applying the renin IRMA after trypsin exposure. This is because of the fact that VTP-27999 will also affect the detection of renin generated by trypsin from prorenin. Therefore, the best way to measure prorenin during VTP-27999 treatment is by making use of the direct prorenin ELISA.

Importantly, although VTP-27999 did not induce prorenin unfolding, it blocked the aliskiren-induced unfolding of prorenin (Figure 3A). This implies that VTP-27999 apparently does bind to the intermediate form of prorenin but that such binding has no conformational consequences. An explanation for the different consequences of inhibitor binding is that aliskiren would be more effective than VTP-27999 at displacing the Ser3 to Asp11 segment from the binding site, which in turn pushes out the prosegment (Figures S5–S8) [9,14,17,18]. The stability of the prosegment conformation is only hinted at by the solution of the prorenin crystal structure (Figure S9), which provides a time- and space-averaged snapshot of the zymogen. Understanding the dynamics of this portion of the prorenin structure would shed light on how these particular inhibitors interact with the protein and vice versa.

Perspectives

VTP-27999 binding to renin increases its immunoreactivity in renin IRMAs, thereby resulting in an overestimation of the renin rise in subjects treated with VTP-27999 by ≈30%. Such changes in immunoreactivity do not occur with aliskiren (Figure 5A) [5]. In addition, VTP-27999 does not induce prorenin unfolding and thus, unlike aliskiren, does not allow the detection of prorenin in a renin IRMA. Aliskiren competitively displaces renin-bound VTP-27999, thereby annihilating the rise in renin immunoreactivity, and allowing a true estimation of the renin rise. Yet simultaneously, aliskiren is capable of converting prorenin to its open, renin-like conformation, thereby resulting in the (indirect) detection of prorenin in renin IRMAs. As a consequence, in plasma samples of VTP-27999–treated subjects, aliskiren may both decrease and increase renin immunoreactivity, and prorenin cannot be determined reliably in such samples. To overcome this problem, a direct prorenin assay is required. Future studies should investigate whether these biochemical differences of VTP-27999 versus other renin inhibitors yield clinically relevant differences. For instance, the lack of effect on the 3D structure of prorenin may reduce the possibility that there is a pool of unfolded

prorenin, which on renin inhibitor dissociation (eg, at trough levels of the drug) would display activity locally. Furthermore, the different binding profile of VTP-27999 may affect renin and prorenin's affinities for their putative receptors, including the mannose 6-phosphate/insulin-like growth factor II receptor and the (pro)renin receptor [19,20].

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Supplemental Information

Aliskiren versus VTP-27999

Aliskiren (Figure S1A) can be considered an inhibitor with an amino-alcohol warhead located near the center of the molecule so that it will interact with renin subsites on the primed and non-primed sides of the binding pocket. VTP-27999 (Figure S1B), on the other hand, uses a secondary amine warhead located at the very end of the molecule so that it only interacts with the non-primed subsites.

In the co-crystal structure of aliskiren bound to human renin solved by Rahuel et al. (RCSB Protein Data Bank accession number: 2V0Z.pdb) [1], there are two monomers in the crystallographic asymmetric unit that differ from one another by the movement of the C-terminal domain closing down over the aliskiren-filled binding pocket (Figure S2A – the overlay of the two aliskiren-bound monomers in the crystallographic asymmetric unit; note the difference between the two monomers at the top right, red to green loop). The average root-mean-square (RMS) deviation between the Cα's of monomer "A" and monomer "B" was calculated to be 0.490A using the LSQ option in the molecular graphics program, Coot [2]. Aliskiren is tucked into the binding pocket of the N-terminal domain and the "flap" (residues Leu73 to Val80) is found in the "closed" conformation, covering the amino-alcohol portion of the inhibitor near the center of the small molecule. Aliskiren occupies the renin subsites S3SP, S3, S1, S1' and S2', where S3SP is a special pocket that projects deeper into the hydrophobic core of the protein from the S3 subsite. Figure S2B is the overlay of the two monomers found in the asymmetric unit of the co-crystal structure of human renin and VTP-27999 (RCSB Protein Data Bank accession code: 3Q4B.pdb). In this case, the average RMS deviation between the Cα's of monomer A and monomer B is calculated to be 0.193A. The conformations of the two inhibited monomers are nearly identical. VTP-27999 occupies the renin subsites S3SP, S3, S2 (barely) and S1. VTP-27999 does not extend into the primed subsites, as noted above. The flap is also found in the closed conformation in this crystal structure.

Figure S2C depicts the overlay of the two monomers (P+A vs. Q+B)6 found in the asymmetric unit of the crystal structure of human prorenin (RCSB Protein Data Bank accession code: 3VCM.pdb). Here the flap is found in the open conformation for monomer Q+B and in a more extended open conformation for monomer P+A. The average

RMS deviation between the Cα's of monomer P+A and monomer Q+B is 0.834A, the largest difference of the three pairs.

Figure S3A is a close-up of aliskiren bound to monomer A (2V0Z.pdb) of human renin and shows a number of H-bonds (green, dashed lines) between protein and inhibitor, especially between the warhead atoms, N22 and O24 and the side chains of Asp32 and Asp215 and the carbonyl of Gly217. The ether oxygen, O2, is H-bonded to the amide nitrogen of Tyr14 in the special pocket, S3SP. Also, the two prime-side residues of aliskiren set up a short antiparallel beta-sheet with local parts of the protein. On the other hand, in the close-up of VTP-27999 bound to monomer A (3Q4B.pdb – Figure S3B), most of the H-bonds between VTP-27999 and the protein are between the warhead atom, N1 and the active site aspartic acids, Asp32 and Asp215. There are two additional H-bonds between VTP-27999 and flap residues Ser76 and Thr77, two more between the carbonyl oxygen of Gly217 and N2 and N4 of the inhibitor and one at the bottom of the special pocket, S3SP, between the amide nitrogen of Tyr14 and O3 of the carbamate.

Figure S4A shows the overlay of these two inhibited structures. The RMS deviation for all Cα's is 0.316A between these two A monomers. Figure S4B is a close-up of the renin active site of the overlaid structures. Notice how the left hand side of the two inhibitors interact with the enzyme; aliskiren goes from subsite 1 directly to subsite 3, while VTP-27999 passes through or close to subsite 2 before dropping into subsite 3.

Inhibited structures versus prorenin

Figure S5 is the overlay of prorenin monomer A with the monomer A's of the two inhibited crystal structures. The flap in the prorenin structure is in an extreme open conformation compared to either of the inhibited structures as a direct result of prosegment residue Met41P and mature renin residues Thr7 and Tyr9 pushing into the area between the flap and the N-terminal domain. The tetrahydropyrane on VTP-27999 also starts to push against flap residues, while aliskiren behaves like a peptidomimetic and allows the flap to close. The two inhibitors follow the trajectory of the prorenin mature chain segment bound in the active site. In Figure S6A it is clear that aliskiren would compete with this segment Val4 to Tyr9. VTP-27999, on the other hand, would directly compete with Leu6 to Tyr9 and maybe the main chain nitrogen of Met10; it would not interfere with binding to any of the prime-subsites (Figure S6B). This can be more clearly seen in Figures S7 (aliskiren versus protein segment Ser3 to Asp11) and S8 (VTP-27999 versus Ser3 to Asp11). These observations suggest that aliskiren would be more effective at displacing the Ser3 to Asp11 segment from the binding site which in turn pushes out the covering propeptide allowing for antibody detection of the exposed propeptide.

A last look at the prorenin structure

Morales et al. [3], pointed out that the prosegment of the P+A monomer could be locked into the observed conformation by crystal lattice contacts, such as the stacking of Trp37P with a symmetry-related Arg74 sequestering the processing site of the

prorenin P+A monomer (which, in solution, should actually be available). This might also be the reason that we see the prosegment of P+A folding over and sitting on the mature renin segment Val4 to Asn8. In Figure S9A, there is a large segment of the Q+B prosegment that is invisible to X-ray analysis because of its suspected mobility due to a lack of crystal lattice contacts as found with the corresponding P+A prosegment. Taking a closer look at the prosegment section that is visible and actually bound in the vicinity of the active site aspartates (Figure S9B), we see a residue "frame-shift" (i.e. compare Leu6A and Leu6B) and as we move from left to right, the prosegments start to go off in different directions. X-ray crystallography provides a time and space-averaged snapshot of the material trapped in a crystal. In this particular case, we have a clue to the dynamic nature of the prosegment of this zymogen by comparing the two monomers present in the crystallographic asymmetric unit. To determine the conformations of the prosegment that are possible in solution, we need to either determine them experimentally by a technique like NMR or in silico by running long time simulations using molecular dynamics. Knowing what conformations are available would result in a better understanding of the interactions of the two renin inhibitors with their prorenin/renin target.

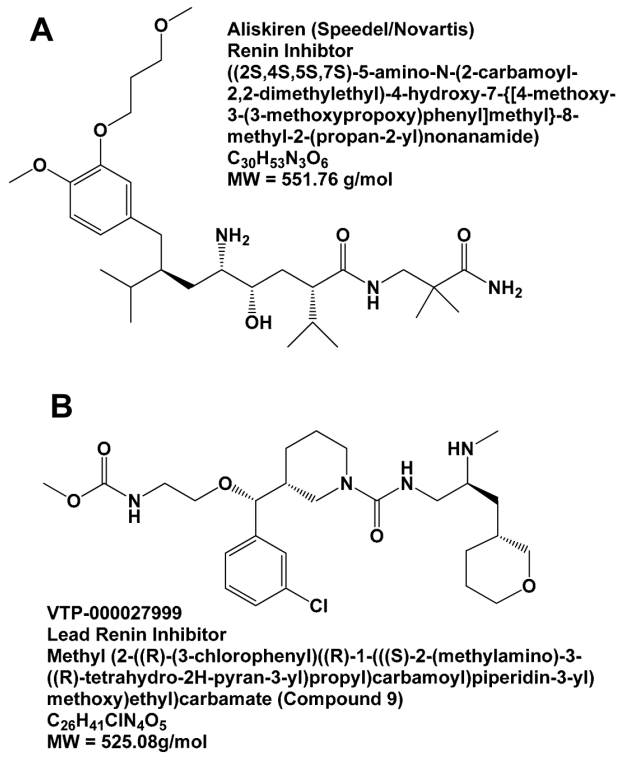
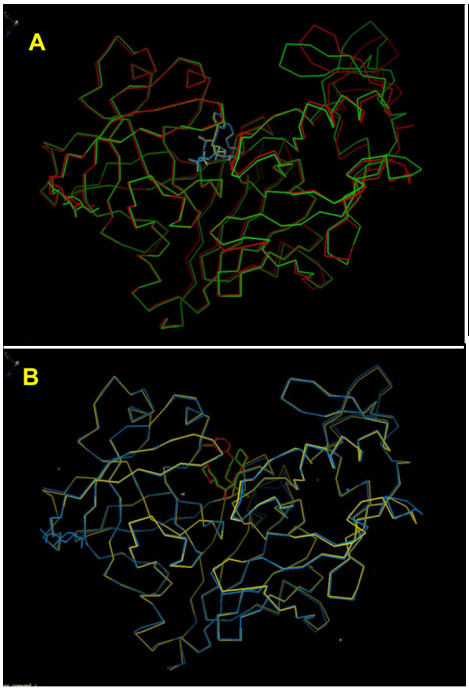


Figure S1. A, The structure of aliskiren. B, The structure of VTP-27999.



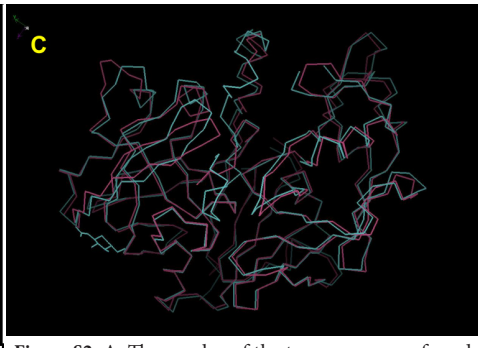


Figure S2. A, The overlay of the two monomers found in the asymmetric unit of the co-crystal structure of human renin and aliskiren (2V0Z.pdb). The proteins are shown as Cα traces with aliskiren given in line format. Monomer A is green and the associated aliskiren molecule blue, while monomer B is colored red with its associated inhibitor colored yellow. B, The overlay of the two monomers found in the asymmetric unit of the co-crystal structure of human renin and VTP-27999 (3Q4B.pdb). Monomer A is blue with the associated VTP-27999 green, while monomer B is colored yellow with its associated inhibitor colored red. C, The overlay of the two monomers found in the asymmetric unit of the crystal structure of human prorenin (3VCM.pdb). Monomer A is cyan, while monomer B is colored magenta.

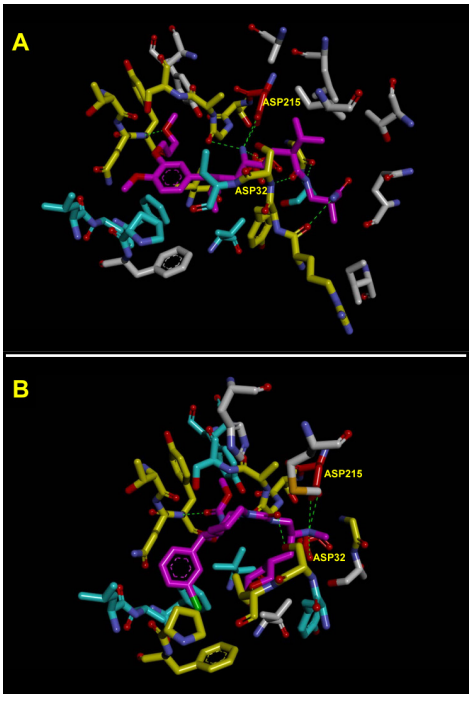


Figure S3. A, Aliskiren (magenta) binding site residues (monomer A). Hydrogen bonds (i.e. interaction distances between nitrogen and oxygen atoms between 2.4A and 3.2A) between aliskiren and renin are shown as green, dashed lines. Asp32 and Asp215 carbons are colored red. Residues with an atom within 3.5A of aliskiren have yellow-colored carbons; within 4.0A have cyan-colored carbons and within 5.0A have white-colored carbons. B, VTP-27999 binding site (monomer A) with residues colored as above.

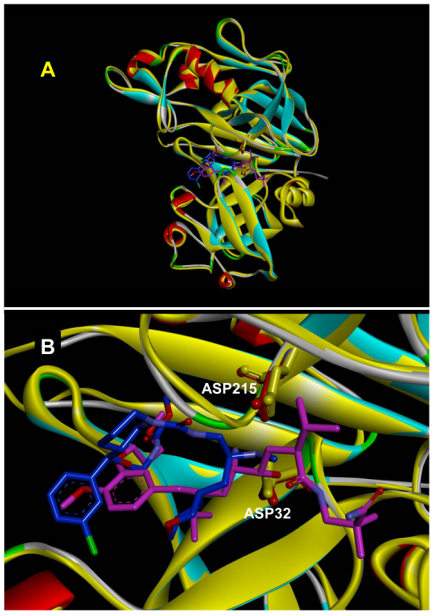


Figure S4. A, Overlay of monomer A from the co-crystal structure of the renin/aliskiren complex with monomer A of the renin/VTP-27999 complex. The protein in the two complexes is represented as a solid ribbon cartoon where the protein in the aliskiren complex is colored as red alpha helices, cyan beta sheet, and green turns and white random coils, while the protein in the VTP-27999 complex is colored yellow for contrast. The active site aspartic acids, Asp32 and Asp215 are shown colored red for the former and yellow for the latter complex. The two inhibitors are rendered in stick format where the carbon atoms of aliskiren are colored magenta and those of VTP-27999 are colored blue. Oxygens are red, nitrogens are gray and chlorine is colored green. B, A close-up of the active site with the flap residues removed for clarity.

Figure S5. A, Side view overlay of renin/aliskiren complex (2VOZ- monomer A- protein cartoon is colored as red alpha helices, cyan beta sheet, green turns and white random coils and stick aliskiren carbons are colored red), renin/VTP-27999 complex (3Q4B.pdb- monomer A- yellow protein and VTP-27999 with blue carbons) and prorenin (3VCM.pdb- monomer A- magenta protein and residue carbons). B, Close-up of the binding pocket.

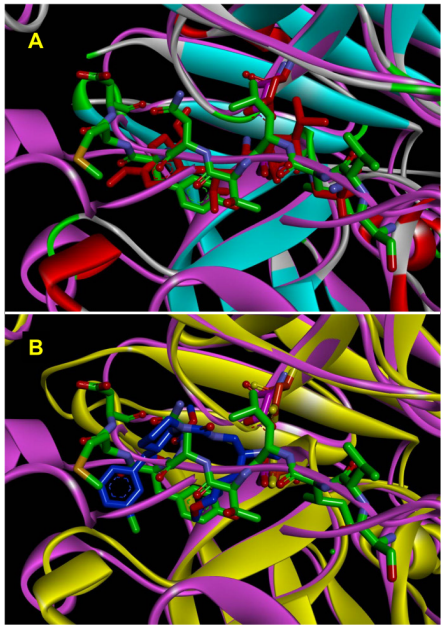


Figure S6. A, Close-up of side view overlay of renin/aliskiren (rendered as in Figure S5) and prorenin (residues now shown with green carbons). B, Close-up of side view overlay of renin VTP- 27999 and prorenin.

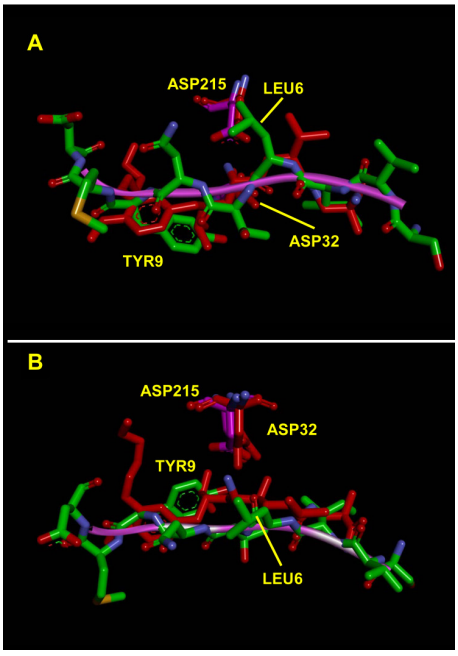


Figure S7. Close-up of the overlay of renin/aliskiren (rendered as in Figure S5) and prorenin (residues shown with green carbons). A, Side view; B, top view.

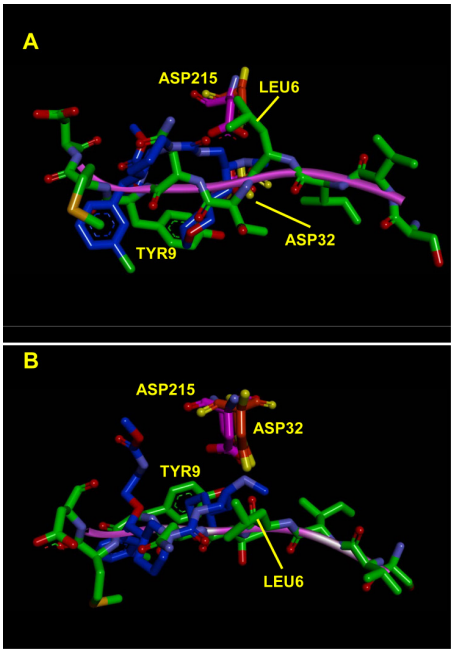


Figure S8. Close-up of the overlay of renin/VTP-27999 (rendered as in Figure S5) and prorenin (residues shown with green carbons). A, Side view; B, top view.

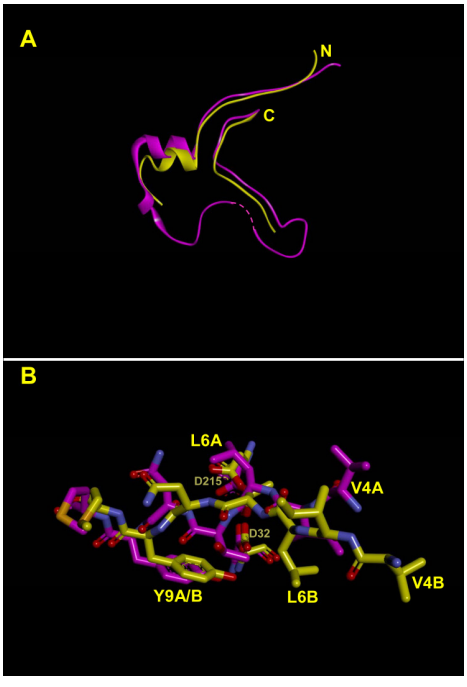


Figure S9. A, Top view of the overlay of residues Thr6P to Glu17 for monomers P+A (magenta) and Q+B (yellow) after least squares superposition of Cα's from prorenin polypeptide B against Cα's of polypeptide A. The dashed curve connects Arg43P with Leu5 of the P+A prosegment. This cannot be done with the Met30Q to Val4 segment of the Q+B prosegment because of the absence of electron density between these two resides. The N and C termini of these segments are indicated. B, Close-up, side view of the residues between Val4 and Met10 following superposition described in A. Carbon atoms colored as in A.

Supplemental Information References

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2. Emsley P, Lohkamp B, Scott W, Cowtan K. Features and development of Coot. Acta Crystallogr D Biol Crystallogr 2010; 66: 486-501.
3. Morales R, Watier Y, Böcskei Z. Human prorenin structure sheds light on a novel mechanism of its autoinhibition and on its non-proteolytic activation by the (pro)renin receptor. J Mol Biol. 2012; 421: 100-111.

Chapter 7

Renin inhibitor VTP-27999 Differs from Aliskiren: Focus on Their Intracellular Accumulation and the (Pro)renin Receptor

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Submitted

Abstract

Background: VTP-27999 is a renin inhibitor (RI) with an IC50 that is comparable to that of aliskiren, but with a higher bioavailability. Unexpectedly, VTP-27999, unlike aliskiren, did not unfold renin's precursor, prorenin, and increased the affinity of the antibodies applied in renin immunoassays.

Methods: Here we verified to what degree these differences affect intracellular RI accumulation in renin-synthesizing human mast cells (HMC-1), and (pro)renin's signalling via the (pro)renin receptor ((P)RR) in rat vascular smooth muscle cells (VSMCs). We also addressed the consequences of (P)RR knockdown by small interfering (si) RNA on (pro)renin release. Finally, making use of FRET(Bodipy-FL)-labeled aliskiren, we studied, by subcellular fractionation, the cellular distribution pattern of this RI.

Results: VTP-27999 accumulated at higher levels in HMC-1 cells than aliskiren, allowing this inhibitor to block intracellular renin at ≈5-fold lower medium levels. Labeled aliskiren accumulated in mitochondria and lysosomes, and its distribution pattern was not identical to that of renin. Moreover, the intracellular accumulation of both inhibitors was unaltered in non-renin synthesizing HEK293 cells, suggesting that it is renin synthesis-independent. VTP-27999, but not aliskiren, blocked renin's capacity to stimulate ERK1/2 phosphorylation in VSMCs, whereas neither inhibitor interfered with prorenin-induced signaling. (P)RR knockdown greatly increased renin (and to a lesser degree, prorenin) release, without affecting the capacity of forskolin or cAMP to stimulate renin release.

Conclusion: VTP-27999 differs from aliskiren regarding its level of intracellular accumulation and its capacity to interfere with renin signalling via the (P)RR, and the (P)RR determines prorenin-renin conversion and constitutive (but not regulated) (pro) renin release.

Introduction

VTP-27999 is a new, active site-directed renin inhibitor with an IC50 (0.3 nmol/L) and half life (30 hours) that are comparable to those of aliskiren (0.6 nmol/L and 40 hours, respectively), but with a much higher bioavailability (>20% vs. 2.6%) [1]. Unexpectedly, VTP-27999 was found to alter the affinity of the active site-directed antibodies applied in renin imunoassays, thereby increasing the outcome of such assays [2]. Such effects on immunoreactivity might also alter renin's binding to clearance receptors and/or the (pro)renin receptor ((P)RR) [3]. In addition, although VTP-27999 did bind to prorenin, it did not, in contrast to aliskiren and other renin inhibitors, subsequently 'unfold' prorenin [2]. Prorenin unfolding normally allows the inactive enzyme to be detected in a renin-specific assay.

To what degree the differences between aliskiren and VTP-27999 have clinical relevance is currently unknown. Theoretically, there might be differences regarding tissue penetration/intracellular accumulation, receptor-mediated clearance and/or (pro)renin receptor binding/signaling. In this study we therefore compared the intracellular accumulation of both inhibitors in renin-synthesizing human mast cells (HMC-1) [4]. Making use of labeled aliskiren, we also investigated in what intracellular compartment, if any, this inhibitor accumulates. This was done because recent studies showed that aliskiren [5,6] and VTP-27999 (R. Gregg et al., Vitae Pharmaceuticals; data not shown) particularly accumulate in the kidney (i.e., a renin-synthesizing organ), and remain present at renal tissue sites long after their concentrations in plasma have decreased to undetectable levels. Furthermore, we studied the effect of VTP-27999 and aliskiren on (pro)renin uptake and (pro)renin-(P)RR-mediated signaling (extracellular signal-regulated kinase 1/2, ERK1/2) in vascular smooth muscle cells (VSMCs) [7,8]. Finally, since mast cells express the (P)RR [9], we evaluated the consequence of (P)RR knockdown by small interfering (si) RNA on (pro)renin release from HMC-1 cells.

Methods

Intracellular accumulation of VTP-27999 and aliskiren

To study whether VTP-27999 and aliskiren accumulate intracellularly (e.g., at renin storage sites), HMC-1 cells (a kind gift of dr. J.H. Butterfield, Mayo Clinic, Rochester, MN) were seeded in 25-cm2 culture flasks at a concentration of 105 cells/mL and cultured for 5 days in 5 mL supplemented Iscove's modified Dulbecco's medium (IMDM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin (Lonza) at 37°C in humidified air with 5% CO2, in the absence or presence of aliskiren or VTP-27999. Next, cells were collected by centrifugation at 1500g for 5 minutes at room temperature, and lysed in 0.2% Triton-X in PBS. Lysates were cleared by centrifugation at 1500g for 10 min at 4°C , and were stored at -20°C. For comparison, similar studies were performed with HEK293 cells (cultured with DMEM medium containing 10% FBS) not synthesizing renin or prorenin.

Subcellular localization of aliskiren

To determine the subcellular localization of aliskiren, 2×107 HMC-1 cells were seeded in 20 mL culture medium, and incubated for 2 days at 37°C with 1 μmol/L FRET(Bodipy-FL)-labeled aliskiren (TFA salt, MW 996.9; a kind gift of dr. Juergen Maibaum, Novartis, Basel, Switzerland). The FRET label is covalently attached by a flexible spacer to the phenyl portion of aliskiren and is not released from parent aliskiren under the experimental conditions of this study. Its IC50 is ≈4 times higher than that of the parent compound (2.5 nmol/L [10]; J. Maibaum, unpublished observations). After incubation, cells were collected, washed twice with ice-cold PBS, and lysed in ice-cold Tris/sucrose buffer (50 mmol/L Tris-HCl, 0.25 mol/L sucrose, 10 mmol/L EDTA, and 3 mmol/L MgCl2) by repeated 5-second

sonication. Subcellular fractionation was performed as described before [11,12]. In short, cell lysate was centrifuged at 1000g for 5 minutes to obtain the nuclear fraction. The supernatant was centrifuged again at 10,000g for 10 minutes to obtain the mitochondrial fraction. The resulting supernatant was centrifuged at 20,000g to obtain the lysosomal fraction. Finally, the remaining supernatant was centrifuged at 180,000g for 30 minutes to separate the microsomal fraction from the cytosol fraction. Pellets were resuspended in Tris/sucrose buffer, and all fractions were stored at -20°C. Fluorescence in each fraction was measured by a plate reader (Molecular Devices) with a standard curve constructed by diluting labeled aliskiren in Tris/sucrose buffer.

(P)RR deletion and (pro)renin release

To study whether the (P)RR affects (pro)renin release, HMC-1 cells were transfected with siRNA using Nucleofector kit L with program T-20 in an Amaxa® Nucleofector® I device following manufacturer's instructions; 5×106 cells and 1 μmol/L siRNA were used per transfection. Silencer® Negative control no.1 siRNA was obtained from Life Technologies. Stealth siRNA against the (P)RR was designed based on the human (P)RR sequence (GenBank Accession No. NM_005765.2), and the sequences were GACAGUGUUGCAAAUUCCAUUCACU (sense) and AGUGAAUGGAAUUUGCAACACUGUC (anti-sense). After transfection, cells were cultured in 2 mL culture medium (1×106 cells/mL) in 6-well plates. At indicated time points, cells and medium were separated by centrifugation at 100g for 10 min at room temperature. Medium was stored at -20°C, and cells were lysed at 4°C in lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 3 mmol/L KCl, 1 mmol/L EDTA, 1% Triton X-100, complete protease inhibitors [Roche], and phosphate inhibitors [Sigma Aldrich], pH 7.4). Lysates were centrifuged at 1000g for 5 min at 4°C, and protein in the supernatants was measured with a BCA assay (Pierce). Equal amounts of protein (20 μg) were separated by SDS-PAGE and transferred to PVDF membranes (Millipore) for immunoblotting. Blots were probed with antibodies against (P)RR/ATP6AP2 (1:1000, Sigma-Aldrich) and β-actin (1:50.000, Millipore). RNA was isolated from cells lysed with Trizol (Invitrogen) to determine gene expression. Validated SYBR green primers for (P)RR were purchased from Promega, and SYBR green primers for renin were designed as follows based on human renin sequence (NM_000537.3): ATGGAACAGAACTCACCCTCCGCT (forward) and ACCGTGATTCCACCCACGGTGAT (reverse). To determine the consequences of (P)RR deletion on stimulated renin release, cells were collected at 48 hours after siRNA transfection, seeded (2×106 cells) in 0.5 mL fresh IMDM medium, and incubated with 50 μmol/L forskolin or 100 nmol/L db-cAMP or their vehicle (DMSO or MQ) for 4 hours at 37°C. Thereafter, medium and cells were collected as described above. Effect of VTP-27999 on (pro)renin-induced signaling and (pro)renin uptake. To study the effect of VTP-27999 on (pro)renin uptake, rat VSMCs were isolated and maintained as described earlier [13], and incubated for up to 24 hours with 4 nmol/L (pro)renin in the presence or absence of 1 μmol/L VTP-27999 and/or 10 mmol/L mannose 6-phosphate (M6P). At the end of the incubation period cells were washed 3 times with 1 ml ice-cold PBS, lysed immediately with 0.2% Triton X-100 in PBS, and stored at -20°C. To determine whether VTP-27999 affects (pro)renin-induced

signaling, VSMCs were incubated at 37°C with 20 nmol/L recombinant human (pro) renin for 5 minutes in the absence or presence of VTP-27999 (1-1000 nmol/L). Incubations with aliskiren (10 μmol/L) were performed for comparison. At the end of the incubation period, the medium was discarded, and the cells were lysed using RIPA lysis buffer, and kept on ice for at least 15 minutes. Next, the cell lysates were centrifuged at 14000g for 15 minutes at 4°C, and supernatants were collected and stored at -20°C. Immunoblotting was performed with 15 μg of protein, using antibodies (Cell Signaling) for phosphorylated p42/44 MAP kinase and total p42/44 MAP kinase (pERK1/2 and ERK1/2 respectively, diluted 1:1000). A peroxidase-conjugated antibody (goat anti-rabbit, 1:5000) was used to visualize pERK1/2 and ERK1/2.

(Pro)renin measurements

Renin and prorenin were measured in medium and cell lysates by enzyme-kinetic assay (EKA) and/or immunoradiometric assay (IRMA; Cisbio). In order to allow its measurement by renin IRMA, prorenin was activated in a non-proteolytic manner by incubating the sample for 48 hours with 10 μmol/L aliskiren at 4°C. Detection limits of the EKA and the renin IRMA were 0.1 ng Ang I/mL per hour and 1 pg renin/mL.

Data analysis

Results are expressed as mean±SEM. IC50 values were calculated as described before [4]. Relative specific activity (RSA) in each subcellular fraction was calculated by dividing the concentration (expressed per mg protein) in the fraction by the concentration (expressed per mg protein) in the homogenate [11]. Ang I-generating activities obtained in the EKA were converted to renin concentrations based on the fact that 1 ng Ang I/mL per hour corresponds with 2.6 pg human renin/mL. Statistical analysis was performed using one-way ANOVA or Student's t-test. . $P<0.05$ was considered significant.

Results

Intracellular accumulation of VTP-27999 and aliskiren

Co-incubation of HCM-1 cells with either aliskiren or VTP-27999 (n=6 each) greatly increased the intracellular levels of immunoreactive renin (Figure 1A). The maximum increases in renin amounted to ≈4-5-fold. Prorenin could not be detected in the cells at any of the tested renin inhibitor concentrations (data not shown). Cellular Ang I-generating activity increased in parallel with immunoreactive renin, except at the highest concentration of VTP-27999, when the cellular Ang I-generating activity dropped below control levels (Figure 1B). The renin activity data should be viewed in the light of the increased immunoreactive renin levels during renin inhibitor exposure. Figure 1C therefore also displays the % of renin inhibitor-bound renin (i.e., blocked renin) as a percentage of the total amount of renin in the cell lysate. It then becomes clear that both renin inhibitors concentration-dependently block intracellular renin. The IC50's (calculated from the inhibitor concentration in the medium, and thus not reflecting the actual inhibitor concentration in the renin-containing granules) amounted to 32±9 nM for VTP-27999 versus 142±35 nM for aliskiren (P=0.02). Thus, ≈5-fold

lower VTP-27999 medium concentrations are required to block 50% of intracellular renin as compared to aliskiren. A similar pattern was observed when incubating the lysates of non-renin-synthesizing HEK293 cells (after their exposure to both inhibitors at increasing concentrations) with a fixed amount of renin (corresponding with ≈200 ng Ang I/ml.hr, n=7 for VTP-27999 and n=4 for aliskiren; Figure 1D). This implies that the intracellular accumulation of VTP-27999 and aliskiren is independent of the renin-synthesizing capacity of the cells, and that the difference between both inhibitors is not related to the fact that VTP-27999 alter renins's immunoreactivity.

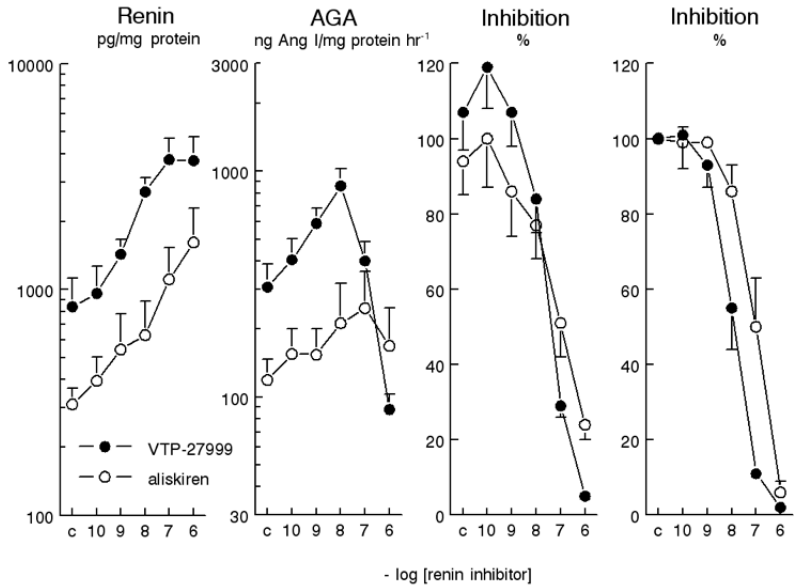

Figure 1. A+B, Cellular immunoreactive renin and Ang I-generating activity (AGA) levels in HMC-1 cells exposed to increasing concentrations of aliskiren or VTP-27999 in the medium. C, % renin inhibitor-bound (i.e., inhibited) renin calculated from the data panels A and B. D, % renin inhibition by lysates of HEK293 cells cultured for 2 days in the presence of increasing concentrations of aliskiren or VTP-27999 (shown on the x-axis). Data are mean±SEM of n=4-7.

Making use of FRET(Bodipy-FL)-labeled aliskiren, we subsequently compared, by subcellular fractionation, the cellular distribution pattern of aliskiren and renin in HMC-1 cells. After a 48-hour incubation, the cells contained 428±28 fmol covalently labeled aliskiren, 539±51 pg renin (≈10 fmol renin) and 6.0±0.1 mg protein (n=3) (Table 1). Recoveries after fractionation were, respectively, 76±9%, 101±12% and 101±8%. Protein levels in the nuclear, mitochondrial, lysosomal, microsomal and cytosolic fractions were 23±5%, 24±1%, 5±1%, 9±3% and 40±6% of the total amount of protein in the homogenate. Relative to protein, aliskiren was low in the cytosolic fraction, and maximally ≈2-fold enriched in the mitochondrial and lysosomal fractions (Figure 2A). Photomicrographs did not reveal substantial enrichment

in a specific cell organelle (Figure 2B). Renin was enriched in the mitochondrial fraction, in agreement with previous studies [14] showing that renin granules can be derived from this fraction, and that they are not present in lysosomes (Figure 2A).

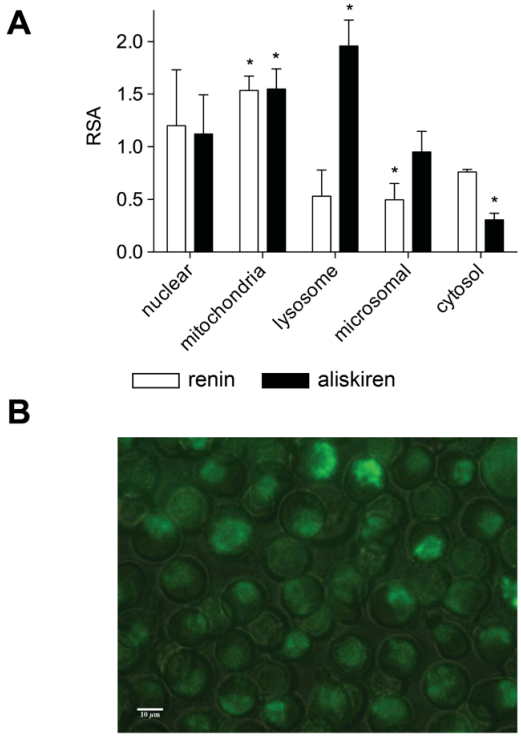

Figure 2. A, Relative specific activity (RSA) of FRET(Bodipy-FL)-labeled aliskiren and renin in subcellular fractions enriched in nuclei, mitochondria, lysosomes, microsomes and cytosol, derived from HMC-1 cells incubated for 2 days with aliskiren. Data are mean±SEM of n=3. *indicates that in all three experiments, the fold changes are either above or below 1.0. B, Photomicrographs (63x oil lens) of live HMC-1 cells following a 2-day incubation with aliskiren; scale bar = 10 μm.

(P)RR and (pro)renin release

(P)RR siRNA decreased the (P)RR protein levels up to 90%, from 18-96 hours after transfection (Figures 3A+3B; n=4). (P)RR mRNA levels, at 48 hours after transfection, were down by 95% (Figure 3C). Thus, (P)RR siRNA nearly completely suppressed (P)RR expression, and this effect lasted at least up to 96 hours after transfection. Following (P)RR knockdown, the release of both renin and prorenin into the medium increased in a time-dependent manner (Figures 4A+4B; n=4 in duplicate), whereas intracellular renin initially decreased (at 48 hours) and then increased in parallel with the medium levels of renin and prorenin (Figure 4C). Intracellular prorenin was undetectable in all samples (data not shown). The rise in (pro)renin was paralleled by a rise in renin mRNA at 48 and 72 hours, which was no longer seen at 96 hours (Figure 4D). Since the effect of (P)RR siRNA on renin release was larger than that on prorenin release, the % of renin (expressed as a % of total renin, i.e., renin + prorenin) in the medium nearly doubled, from 30±3% to 57±2% ($P<0.05$). To study the effect of (P)RR knockdown on regulated renin release, cells were exposed to forskolin or db-cAMP (n=2-3 triplicate measurements). The % increases (≈20-30%; Figures 4E+4F) were identical with both stimulants, and unaltered by (P)RR knockdown.

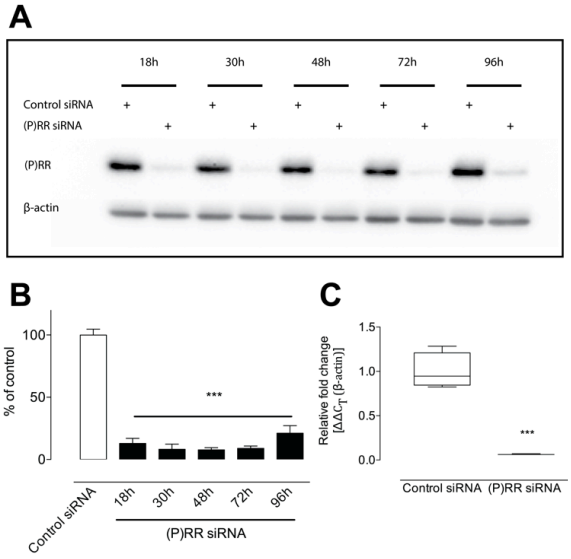

Figure 3. (P)RR siRNA, but not control siRNA, transfection of HMC-1 cells suppresses (P)RR expression from 18 to 96 hours after transfection (A, representative Western blot of cell lysates versus β-actin; B, (P)RR protein, corrected for β-actin, expressed as a % of control; C, (P)RR mRNA expression, 48 hours after transfection). Data are mean±SEM of n=3, ***, P<0.001.

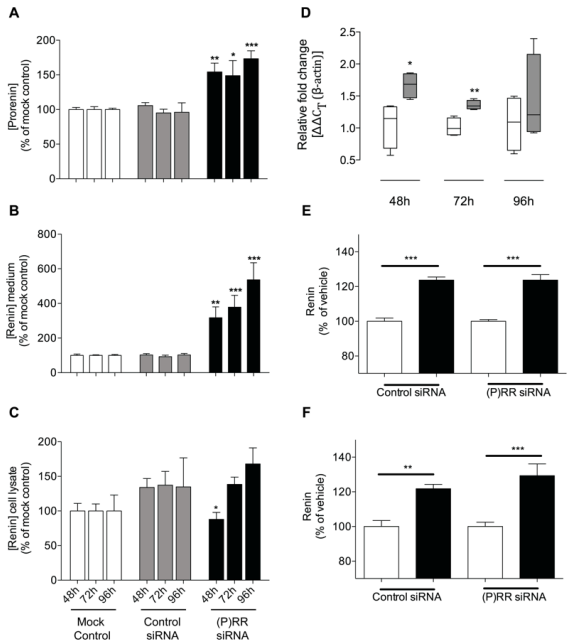

Figure 4. A-C, (Pro)renin levels in medium and cell lysates of HMC-1 cells transfected with control or (P)RR siRNA at 48, 72 or 96 hours after transfection, expressed as a % of mock-transfected cells. Medium prorenin and renin levels in mock-transfected cells at 48, 72 and 96 hours were 19.6±4.1, 31.5±10.4, and 36.8±11.1 versus 9.6±1.6, 13.1±3.3 and 14.5±4.1 pg/mg protein, whereas the accompanying cellular renin levels were 281±31, 356±36 and 214±49 pg/mg protein, respectively. Data are mean±SEM of 4 duplicate measurements; *P<0.05, **P<0.01, ***P<0.001 vs. control siRNA. D, Renin gene expression in HMC-1 cells 48-96 hours after transfection (mean±SEM of 2 duplicate measurements). Open bar: control siRNA treated; Gray bar: (P)RR siRNA treated. *P<0.05; **P<0.01 vs. control siRNA. E+F, Medium renin levels after a 4-hour stimulation of HMC-1 cells with vehicle, forskolin (D) or db-cAMP (E). Renin levels in control siRNA and (P)RR siRNA treated cells receiving vehicle were 6.7±1.5 versus 10.8±1.9 (D), and 7.1±2.3 versus 8.4±2.3 pg/mg protein, respectively (E). Open bar: Vehicle; Closed bar: forskolin or db-cAMP. Data are mean±SEM of 2-3 triplicate measurements; **P<0.01; ***P<0.001 vs. vehicle.

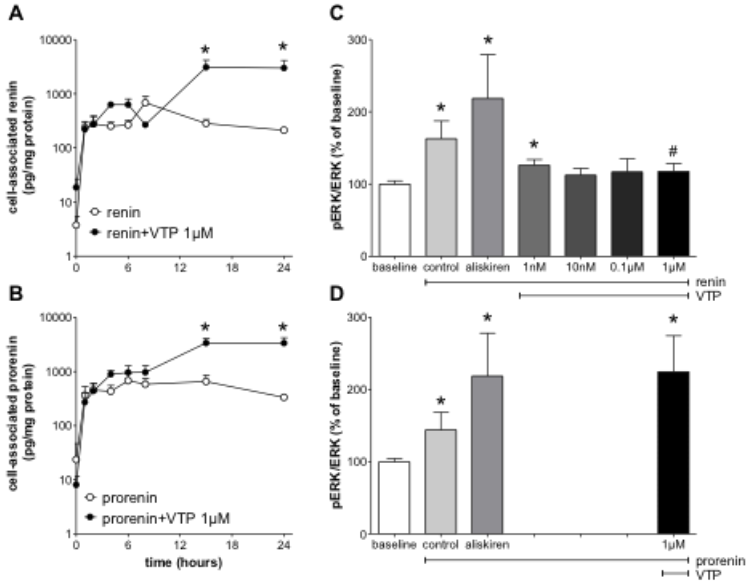

Figure 5. A+ B, Cell-associated (pro)renin levels after incubation of VSMCs with 4 nmol/L (pro)renin in the absence or presence of 1 µmol/L VTP-27999; *P<0.05 vs. no VTP-27999. C+D, Cellular pERK1/2/ERK1/2 ratios after a 5-minute incubation of VSMCs with 20 nmol/L (pro)renin in the absence (control) or presence of aliskiren or VTP-27999;* P<0.01 vs. baseline, # P<0.05 vs. control. Data are mean±SEM of n=5-8

Effect of VTP-27999 on (pro)renin-induced signaling and (pro)renin uptake

Incubating VSMCs with renin or prorenin at 37°C resulted in a rapid accumulation of both proteins in the cells, reaching a maximum after 2-4 hours (Figures 5A+5B; n=5 for both). In the presence of 1 µmol/L VTP-27999, a steady-state was reached after 12-18 hours, and the cell-associated levels under these conditions were up to 14-fold higher than without VTP-27999 (Figure 5). M6P blocked the renin/prorenin accumulation, both with and without VTP-27999, by maximally 55% (data not shown), indicating that the cellular uptake, at least partly, depended on M6P receptor-mediated internalization of both renin and prorenin. This fully resembles our previous observations [12].

Both renin and prorenin induced ERK1/2 phosphorylation in VSMCs (Figures 5C+5D; n=8). In agreement with previous studies, aliskiren did not prevent this phosphorylation. VTP-27999 also did not prevent the phosphorylation induced by prorenin, but concentration-dependently did block the ERK1/2 phosphorylation induced by renin (P<0.05).

Discussion

This study shows that the extracellular levels of VTP-27999 that are required to block intracellular renin in renin-synthesizing HMC-1 cells are ≈5-fold lower than those of aliskiren. In agreement with this finding, the renal tissue/plasma concentration ratio of VTP-27999 in rats at 24 hours after oral dosing (10 mg/kg) was 6-fold higher than that of aliskiren at 10 mg/kg (R. Gregg, Vitae Pharmaceuticals, unpublished observations). Such accumulation, at least in the case of aliskiren, was characterized by a modest enrichment in mitochondria and lysosomes. Although renin was also found in the former, the VTP-27999/aliskiren accumulation difference was identical in non-renin synthesizing HEK293 cells, suggesting that intracellular renin inhibitor accumulation is independent of the renin-synthesizing capacity of cells. Indeed, in vivo, aliskiren accumulation occurred to the same degree in kidneys of renin knockout animals as in kidneys of wild-type animals [5]. Moreover, in the current study, on a molar basis, the amount of cell-associated aliskiren was >40 times the level of renin, suggestion that >95% of aliskiren accumulation was renin-independent. Nevertheless, such accumulation did allow the release of renin inhibitor-bound renin from HMC-1 cells, even when stimulated with forskolin in the absence of the renin inhibitor [4], and could thus explain the long-lasting suppression of PRA following treatment discontinuation [15]. Unfortunately, since we had no access to labeled VTP-27999, fractionation studies with this compound could not be performed. Furthermore, even the data obtained with labeled aliskiren should be interpreted with care, since we cannot exclude that the FRET label might somehow have affected its subcellular distribution.

Exposure to VTP-27999 or aliskiren increased renin immunoreactivity in HMC-1 cells 4-5-fold. This is much higher than the modest rise (30-40%) expected in the case of VTP-27999 due to its capacity to alter the affinity of the active site-directed antibodies applied in renin imunoassays [2]. Clearly therefore, these rises truly reflect an increase in the absolute levels of renin. As a consequence of these rises, the cellular Ang I-generating capacity did not decrease until the medium renin inhibitor concentrations exceeded 10 nmol/L. In vivo, such a rise in renin would be attributed to interference with the negative feedback loop between Ang II and renin release. However, this cannot be the explanation for the rise in intracellular renin in HMC-1 cells, since HMC-1 cells do not generate angiotensinogen and thus cannot synthesize angiotensins [4]. We have demonstrated that, in the case of aliskiren, it is related to the increase in half life of renin when bound to the renin inhibitor, due to the fact that aliskiren-binding stabilizes the molecule [12]. Apparently, renin binding to VTP-27999 has the same consequence. Indeed, when incubating non-renin synthesizing rat VSMCs with renin, this resulted in much higher cell-associated renin levels in the presence of VTP-27999 than without this drug. Identical findings were obtained previously for aliskiren [12]. Moreover, it took up to 18-24 hours before a steady-state cellular renin level had been reached, as opposed to a few hours in non-renin inhibitor-exposed cells, in full agreement with a much higher half life of renin inhibitor-bound renin. The greater accumulation in the presence of VTP-27999 was also observed for prorenin, although VTP-27999 binding does not alter

the 3D structure of prorenin (and therefore its half life) [2]. However, once internalized, prorenin is rapidly converted to renin by actual cleavage of the prosegment, thereby still allowing enhanced intracellular accumulation via binding to intracellular VTP-27999 [16]. The internalization of renin and prorenin in VSMCs is mediated via M6P receptors, which act as clearance receptors for both proteins [3,12]. Indeed, excess M6P greatly reduced the cellular (pro)renin accumulation in the present study. There is no role for the (P)RR in this process, as the results were identical in human (P)RR-overexpressing rat VSMCs (n=5; data not shown) and wild-type rat VSMCs. This is due to the fact that (pro)renin bound to membrane (P)RR does not internalize [13].

Recent studies have indicated an association between the (P)RR and vacuolar-type H^+-ATPase (V-ATPase) [17-19]. V-ATPases are multisubunit proteins that consist of a V0 proton-translocation domain, a V1 pump domain and two associated proteins, Ac45 and the (P)RR. V-ATPases are found in virtually all cell types, mostly on the membranes of intracellular compartments, and are important for vesicle trafficking, protein degradation and coupled transport [20]. V-ATPase-mediated acidification of intracellular compartments is crucial for multiple cellular events, including the proteolytic processing of proinsulin to insulin. To investigate whether it also determines prorenin-to-renin conversion, we studied the consequences of (P)RR knockdown on (pro)renin secretion in HMC-1 cells. Our data revealed that the basal release of renin, and to a lesser degree of prorenin, increased in the absence of the (P)RR. Although initially resulting in a modest reduction in cellular renin, the increase in renin expression observed at 48 and 72 hours after transfection rapidly made up for this phenomenon, and allowed a continuation of the increased basal (pro)renin release in the face of unaltered cellular renin levels. As a consequence of the larger effect on renin, the percentage of total renin (=renin + prorenin) released in the active form doubled. These data suggest that the (P)RR indeed determines prorenin-renin conversion, as well as constitutive (pro)renin release. (P)RR deletion however did not alter the forskolin- or db-cAMP-induced release of renin, thereby excluding a role for this receptor in cAMP-induced renin exocytosis.

In some cell types, V-ATPases are also abundantly present at the plasma membrane, for example in intercalated cells of the collecting duct, where they regulate systemic acid-base homeostasis [21]. In such cells, prorenin directly stimulated V-ATPase activity at pmolar levels in a (P)RR-dependent manner [22]. Higher (nanomolar) levels were required to induce ERK1/2 phosphorylation via (P)RR stimulation in VSMCs [8], most likely because the receptors in these cells are less abundant on the cell surface and/or largely located intracellularly. In agreement with previous studies [8,23], we observed that both renin and prorenin stimulated ERK1/2 phosphorylation in rat VSMCs at nanomolar levels, and that aliskiren did not block this effect. Apparently therefore, aliskiren binding to renin or prorenin does not interfere with their binding to the (P)RR. Yet, VTP-27999 did concentration-dependently block the effect of renin on ERK1/2 phosphorylation, and did not interfere with the prorenin-induced effects. These data parallel our previous observations demonstrating that VTP-27999 binding

to prorenin does not induce prosegment unfolding (i.e., does not alter the 3D structure of prorenin) [2], whereas binding to renin altered its affinity for active site-directed monoclonal antibodies. Apparently, VTP-2999-binding to renin, in addition to affecting its clearance and binding to antibodies, also prevented renin from binding to the (P)RR. These data are the first to demonstrate that a renin inhibitor is capable of blocking the (P)RR-induced effects of renin. This capacity further distinguishes VTP-27999 from aliskiren.

Conclusion

Not all renin inhibitors are equal. In comparison to aliskiren, VTP-27999 accumulates at higher levels in renin-synthesizing cells, thus potentially allowing the release of more inhibitor-bound renin, even when the extracellular levels of the renin inhibitor have decreased to zero. VTP-27999 binding to renin affects its clearance and binding to antibodies, but also its capacity to induce signaling via the (P)RR. This does not apply to prorenin, most likely because VTP-27999 binding to prorenin does not alter its 3D structure. Moreover, such blocking effects towards renin-induced signaling were not observed for aliskiren. To what degree these biochemical differences between both renin inhibitors result in meaningful clinical differences now needs to be established in clinical trials.

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Chapter 8

Summary and General Discussion

Summary and General Discussion

The function of prorenin, the precursor of renin, remained unknown until the discovery of the (pro)renin receptor [(P)RR]. (Pro)renin binding to the (P)RR leads to angiotensin generation and induces both angiotensin II–dependent and –independent intracellular signaling. The concept of (pro)renin acting as a (P)RR agonist, inducing signaling angiotensin-independent signaling, is in the center of debate. Data obtained from transgenic animals with excessive prorenin levels suggest that prorenin exerts its effects by angiotensin II generation [1, 2], and the phenotype of (P)RR transgenic animals does not show alterations in RAS components [3, 4]. Although in vitro studies indeed support (pro)renin-induced intracellular signaling via the (P)RR, it must be noted that the (pro)renin concentrations applied in these studies are in the nanomolar range, which is several magnitudes higher than the physiological (pro)renin levels [5-7]. Even under pathological conditions, for example in diabetes mellitus, plasma prorenin levels are at most 5- to 10-fold increased, which is still insufficient for direct (P)RR signaling [8, 9]. In addition, the (P)RR has been identified as an accessory protein of the V-ATPase complex, playing a crucial role in Wnt/β-catenin signaling and maintaining V-ATPase integrity, independent of (pro)renin [10, 11]. It is thus clear that the (P)RR has important functions completely unrelated to the RAS. In fact, (P)RR knockout, as opposed to RAS component knockout, is lethal, even when limited to cells such as cardiomyocytes and podocytes [12-14]. Therefore, the physiological relevance of (pro)renin-(P)RR interaction remains unclear and needs to be carefully evaluated. We, thus, set out to investigate the effects of (pro)renin-(P)RR interaction and the (pro)renin-independent (P)RR function(s).

In **Chapter 2**, we studied (pro)renin-(P)RR interaction in vitro using rat vascular smooth muscle cells (VSMCs) expressing the human (h) (P)RR. In combination with recombinant human prorenin, it allowed us to study if (pro)renin-(P)RR interaction occurs. We found that VSMCs expressing the h(P)RR, when incubated with human prorenin, sequester a significantly higher amount of human prorenin as compared to wild type VSMCs, and this effect was eliminated by knocking down the h(P)RR using siRNA. This is an important piece of evidence directly showing that prorenin, at least at the concentration tested (80 nmol/L), binds to the (P)RR. In addition, 20 nM prorenin exclusively stimulated Erk1/2 phosphorylation in VSMCs expressing h(P)RR, and this effect was eliminated by h(P)RR targeting siRNA. Lack of angiotensinogen expression in VSMCs excludes the possibility that the observed Erk1/2 activation is due to angiotensin II generation. Yet, the prorenin concentration required for direct (P)RR signaling is several orders of magnitude above the normal plasma levels in vivo. Such prorenin levels are not likely to occur in vivo in non-(pro)renin synthesizing tissues.

The (pro)renin concentrations required for (pro)renin-(P)RR interaction and direct (P)RR signaling may differ in different cell types. Advani et al. showed that, in collecting duct cells, both renin and prorenin are able to stimulate Erk1/2 phosphorylation at concentration as low as 20 pM [10]. This concentration is about 4-fold higher for prorenin and 40-fold higher for renin than that in normal subjects (~5 pM and ~0.5

pM respectively). It is also noticeable that (pro)renin- stimulated Erk1/2 activation in collecting duct cells requires V-ATPase activity. Blocking V-ATPase activity using bafilomycin A1 completely blocked (pro)renin-induced Erk1/2 activation. Moreover, the (P)RR has been shown to colocalize with V-ATPase in collecting duct cells. Taken together, this suggests that V-ATPase facilitates (P)RR signaling, and the possibility exists that in cells in which (P)RR colocalizes with V-ATPase on the cell surface, lower levels of (pro)renin are required for angiotensin-independent (P)RR signaling.

V-ATPases are expressed in virtually all cell types in intracellular compartments. In special cells, like the intercalated cells of the collecting duct, V-ATPases are also expressed at the plasma membrane. Interestingly, the (P)RR is also strongly expressed in intercalated cells of the collecting duct and colocalizes with V-ATPase at the luminal membrane [10]. Thus, the collecting duct is a very likely place where (P)RR-V-ATPase interaction occurs and where relatively low levels of (pro)renin might be sufficient to initiate signaling via the (P)RR. In addition, both (P)RR expression and prorenin secretion are increased in the collecting duct in diabetes mellitus, raising the possibility that indeed under certain (diabetic) conditions locally sufficiently high prorenin levels may be reached to bind to the (P)RR [8, 15-17]. Furthermore, the question remains whether prorenin-(P)RR interaction directly regulates V-ATPase activity. We thus studied the role of the (P)RR in V-ATPase regulation.

In **Chapter 4**, we found that prorenin stimulates V-ATPase activity dose-dependently in MDCK.C11 cells. These cells resemble the intercalated cells of the collecting duct. In the range tested (10 pM to 10 nM), prorenin significantly stimulated V-ATPase activity at 1 nM and 10 nM. The effect of prorenin on V-ATPase is independent of angiotensin II generation, as the presence of the angiotensin II type 1 and 2 (AT1, AT2) receptor blockers irbesartan and PD123319 did not alter prorenin-stimulated V-ATPase activity. In addition, depletion of the (P)RR using siRNA completely blocked prorenin-stimulated V-ATPase activity, suggesting that the effect is indeed mediated by the (P)RR. However, the concentration required, though 20-fold less than that for stimulating Erk1/2 in VSMCs, is still more than 100 times higher than the prorenin levels in normal subjects. Whether this concentration is reachable or not at the level of the collecting duct is important to validate the physiological relevance of (pro)renin-(P)RR interaction for V-ATPase regulation.

There are two major renal sources for (pro)renin: juxtaglomerular cells and principal cells of the collecting duct [18]. Elevated plasma prorenin levels are firmly associated with the progression of diabetes. Furthermore, a rat diabetic model suggests that the collecting duct is the major source for the increased prorenin levels in this disorder [19]. However, it is not yet clear if increased prorenin in patients with diabetes mellitus is derived from collecting duct. We thus, in **Chapter 3**, studied urinary renin and prorenin in patients with type 2 diabetes (T2D) following a 2 month treatment period with placebo, the renin inhibitor aliskiren, the AT1 receptor blocker irbesartan or aliskiren + irbesartan. Although T2D patients have elevated plasma levels of

prorenin, we were unable to detect prorenin in the urine samples. Following treatment with aliskiren, irbesartan, or their combination, plasma renin increased 2.5-10 fold, as compared to placebo, while urinary renin levels remained unchanged. This leads to decreased urinary to plasma renin ratios. Our study suggests two likely possibilities where urinary renin is originated. First, the renin may originate exclusively in the kidney from a source that is regulated independently from the juxtaglomerular cells. Second, urinary renin may originate both in the kidney and blood. Despite the possibility that the collecting duct may synthesize (pro)renin under pathologic conditions, its concentration, as indicated by the urinary renin concentration and the absence of urinary prorenin, appears to be far below the supraphysiological concentrations required for (pro)renin-(P)RR interaction and signaling. However, it is also worth noticing that the site of (pro)renin synthesis and secretion in the collecting duct is in principal cells, which are adjacent to intercalated cells. Thus, it is still possible that the local (pro) renin levels are high enough to regulate V-ATPase activity in a paracrine manner.

Despite the controversy regarding (pro)renin-(P)RR interaction, the (P) RR itself (i.e., independently of (pro)renin) has been implied to play a role in cardiovascular complications. (P)RR overexpression is upregulated under various pathological conditions, for example in the heart and kidney of rats with congestive heart failure, in the heart of stroke-prone spontaneously hypertensive rats (SHRsp) on a high salt diet, in kidneys of rats with end stage diabetic nephropathy, in remnant kidney of uninephrectomized rats, and in the clipped kidney of Goldblatt hypertensive rats [16, 17, 20-22]. Furthermore, overexpression of the (P)RR, without altering RAS component levels, resulted in increased blood pressure, increased plasma aldosterone levels, increased renal cyclooxygenase 2 expression, and glomerulosclerosis [3, 23]. Blocking the (P)RR may therefore provide beneficial effects in preventing or slowing down cardiovascular complications. A peptidic antagonist was designed based on the interaction between the prorenin 'handle region' and (P)RR [24-26]. This handle region peptide (HRP) mimics a 10 amino acid region of the prosegment of prorenin, which is believed to be important in prorenin binding to the (P)RR. Although beneficial effects of HRP have been reported in animal models, several studies also suggest HRP may act as a partial agonist of the (P)RR, or that it might have (P)RR-independent effects [27-32].

We thus examined the effects of the HRP on (pro)renin-(P)RR signaling. In **Chapter 2**, we found that HRP did not affect the DNA synthesis induced by (pro)renin + angiotensinogen in VSMCs, suggesting that it does not affect prorenin activation by (P)RR. Furthermore, 1 μM HRP did not alter renin-induced Erk1/2 activation. This suggests that HRP exerts no blocking effects towards either angiotensin-dependent and –independent (P)RR signaling. In **Chapter 4**, we further studied the effect of HRP on (pro)renin-(P)RR signaling in relation to V-ATPase regulation. Consistently, 1 μM HRP did not block 10 nM prorenin-induced V-ATPase activity in MDCK.C11 cells. Strikingly, HRP in fact stimulated V-ATPase activity in a dose-dependent manner. (P)RR depletion completely blocked HRP-induced V-ATPase activity, suggesting that HRP stimulates V-ATPase in a (P)RR dependent manner. Clearly, our data reveal an

unexpected agonist function of HRP. In fact, other researchers reported stimulatory effects of HRP on Erk1/2 activation as well, in line with our finding that HRP may act as a (P)RR agonist [33, 34]. This, however, contradicts previous reports stating that HRP has beneficial effects on end-organ damage in disease models via its (P)RR-blocking capacity. In fact, other studies also challenged the claimed beneficial effects of HRP, as it did not improve nephrosclerosis in Goldblatt rats and even counteracted the beneficial effects of renin inhibition in spontaneously hypertensive rats [32]. It remains difficult to explain the discrepancies among the observed effects of HRP, especially due to lack of a complete understanding of the (P)RR function(s).

In the past few years, the (P)RR has been reported to play important roles in Wnt signaling and V-ATPase integrity, independent of (pro)renin. In **Chapter 4**, we found that depletion of the (P)RR did not affect basal V-ATPase activity, but eliminated vasopressin-stimulated V-ATPase activity, demonstrating that the (P)RR also regulates V-ATPase independent of prorenin. Furthermore, we found that (P)RR depletion did not, as described in previous studies, downregulate all Vo subunits, but selectively downregulated Voa2. In the kidney, Voa4 is the most abundant and dominant Voa subunit, and it predominantly expresses at the plasma membrane. These data thus explain why basal V-ATPase activity was unaffected by (P)RR depletion while stimulated V-ATPase activity was eliminated. Our data highlight a prorenin-independent function of the (P)RR in V-ATPase regulation. Although the (P)RR is important for V-ATPase integrity and regulation, little is known about the function of (P)RR-V-ATPase interaction in the cell. Recently, Cruciat et al. found that the (P)RR acts as an adaptor between Frizzled coreceptor LRP6 and V-ATPase, and is indispensible for Wnt signaling [11]. This suggests that the (P)RR may act as an adaptor between V-ATPase and other receptors/proteins, thereby regulating different cellular processes.

In **Chapter 5**, we used a proteomics approach to identify (P)RR-interacting proteins in an unbiased manner. N-terminal TAP tagged (P)RR was successfully purified from HEK293 cells, and co-purified proteins were identified by mass spectrometry. In this approach, we found, not surprisingly, that several V-ATPase subunits co-purified with the (P)RR, including the previously described VoD and VoC subunits. This strongly supports the efficacy of our method. Additionally, we found several hundred other (P)RR-interacting proteins, which were involved in signal transduction and lipid metabolism, protein folding/modification, and cytoskeleton structure. Among these potential binding partners of the (P)RR was sortilin-1. We validated this interaction using immunoprecipitation. Furthermore, we also observed that sortilin-1 and (P)RR mutually regulate each other's protein abundance: (P)RR depletion leads to decreased sortilin-1 expression, and vice versa. (P)RR depletion did not affect sortilin-1 mRNA expression, nor did sortilin-1 depletion affect (P)RR mRNA expression, suggesting that the effect on protein abundance is post-transcriptional.

Sortilin-1, also known as neurotensin receptor 3 and encoded by the SORT1 gene, is a multi-ligand sorting receptor. Sortilin-1 is highly expressed in neurons, regulating neuron viability and functions [35]. Furthermore, studies have demonstrated that sortilin-1 controls intracellular trafficking of APP, the precursor of amyloid-β (Aβ) which is the major component of senile plaque found in the brain of Alzheimer patients [36]. Sortilin-1 binds to ApoE with high affinity, and is important for neuron clearance of ApoE/Aβ. This suggests an intriguing role of sortilin-1 in the progression of Alzheimer's disease. Sortilin-1 is also involved in the formation of Glut4 storage vesicles, and has been reported to mediate WNK4-induced lysosomal degradation of NCC [37, 38]. Interestingly, in recent GWAs studies, genetic variances in SORT1 have been shown to associate with LDL-C levels [39, 40]. Further studies revealed that sortilin-1 is a clearance receptor for plasma LDL which regulates lipid homeostasis by controlling ApoB secretion and LDL uptake [41]. These data suggest that, by controlling the abundance of sortilin-1, the (P)RR may also play a role in multiple cellular processes. This may in fact explain why (P)RR deletion is lethal even when limited to certain tissues, while global knockout of RAS components has no such effect.

Direct renin inhibitors are a new class of antihypertensive drugs aiming at blockade of the renin-angiotensin system. Aliskiren is the first approved drug in this class for clinical use. At approved doses, it decreases blood pressure in a dose-dependent manner. Recently, a new direct renin inhibitor, VTP-27999, has been developed. It has a similar IC50 and half-life as aliskiren, but displays a nearly 10 times higher bioavailability. We compared this inhibitor with aliskiren in **Chapters 6 & 7**. VTP-27999, like aliskiren, acutely increased renin levels by up to 20-fold, and increased it >100-fold on day 10, when given at increasing doses to healthy volunteers on a low salt diet. Plasma renin levels decreased after stopping the treatment, with a half-life of 39±10 hours in aliskiren-treated and 30±3 hours in VTP-27999-treated healthy volunteers, in line with the half-life of these two inhibitors. In addition to renin binding, aliskiren can also bind to prorenin. This leads to activation of prorenin in a non-proteolytic manner (i.e., prorenin unfolding), which subsequently allows the measurement of prorenin in a renin immunoradiometric assay (IRMA). Yet, in contrast to aliskiren, we found that VTP-27999 binds to prorenin without inducing prorenin unfolding. Further tests showed that both VTP-27999 and aliskiren are able to lock acid-activated prorenin in its open conformation, allowing its detection by renin IRMA. Surprisingly, we also observed that VTP-27999 binding caused an increase in renin immunoreactivity as measured by the active site-directed antibody, suggesting it alters the affinity of the antibody. This has not been seen for aliskiren. The differences between VTP-27999 and aliskiren suggest that they may show differences in tissue penetration/intracellular accumulation, and that they may have different effects on (pro)renin-(P)RR interaction.

Therefore, we studied intracellular accumulation of aliskiren and VTP-27999 in renin-synthesizing human mast cells (HMC-1). We found that VTP-27999 accumulates in HMC-1 cells at ≈5-fold higher levels than aliskiren. Importantly, such accumulation differences were also observed in non-renin-synthesizing HEK293 cells, which suggests

that the aliskiren and VTP-27999 accumulation is independent of renin. Our findings, at least in the case of aliskiren, are in line with a previous study which showed that aliskiren accumulation in the kidney is not related to (pro)renin [42]. Using fluorescently labeled aliskiren we observed a modest enrichment of aliskiren in mitochondria and lysosomes, independently of renin. As mentioned above, VTP-27999 alters renin immunoreactivity detected by active site-targeted antibodies. This alteration is likely caused by changes in renin conformation upon VTP-27999 binding, which facilitate the exposure of active site to the antibody. Such changes may, in theory, also affect renin clearance via mannose 6-phosphate receptors (M6PR). In fact, we found that, in the presence of VTP-27999, (pro)renin accumulation in VSMCs increased ≈14-fold as compared to (pro) renin uptake without VTP-27999. M6P blocked the renin/prorenin accumulation in VSMCs by up to 55%, regardless of the presence of VTP-27999, confirming that the M6PR is the major receptor responsible for (pro)renin uptake. Thus, our data suggest VTP-27999 diminishes intracellular (pro)renin breakdown, likely by altering the renin conformation. Holding this view on VTP-27999, we further studied if it affects (pro)renin-(P)RR interaction and signaling. Both renin and prorenin induced Erk1/2 phosphorylation in VSMCs, and aliskiren did not affect this phosphorylation, in line with previous study [43]. However, VTP-27999, although it did not alter prorenin-induced Erk1/2 activation, concentration-dependently blocked renin-induced Erk1/2 activation. This confirms that VTP-27999 may indeed affect the renin conformation, thereby altering the interaction between renin and its receptors [M6PR and (P)RR].

(P)RR interacts with V-ATPase and plays an important role in regulating V-ATPase activity. V-ATPase-mediated acidification of intracellular compartments is crucial for multiple cellular events, including the proteolytic processing of proinsulin to insulin [44, 45]. An acidic environment will move the prosegment of prorenin out of its enzymatic active pocket, thereby facilitating its cleavage. We thus hypothesized that (P)RR-V-ATPase may determine prorenin-to-renin conversion. Hence, we studied the effect of (P)RR knockdown on (pro)renin secretion in HMC-1 cells. We found that the basal release of renin, and to a lesser degree of prorenin, increased by depleting the (P)RR using siRNA, in a time-dependent manner. This effect was associated with an initial decrease in cellular stored renin, and increased renin mRNA level, 48 hours after knocking down (P)RR. As a result of this increased renin expression, the cellular renin levels were back to normal at 72 and 96 hours, and the renin mRNA levels returned to normal levels at 96 hours after (P)RR depletion. These results suggest that depletion of the (P)RR results in an acute renin release from storage, while the fall in stored renin leads to increased renin expression as a compensatory mechanism to maintain steady state renin storage. (P)RR depletion did not alter forskolin- or db-cAMP-induced renin release, excluding a role of this receptor in cAMP-induced renin exocytosis. Overall, our study reveals that the (P)RR determines prorenin-to-renin conversion and controls constitutive renin release.

Future Perspectives

In **Chapters 2 to 4**, we investigated (pro)renin-(P)RR interaction in different cell models, as well as the (pro)renin levels required for such interaction. It turned out that the (pro)renin levels required for both for the angiotensin–dependent and –independent (P)RR functions were several orders of magnitude higher than the (pro)renin levels that occur in vivo. It is thus unlikely that (pro)renin-(P)RR interaction and signaling will occur in vivo except in (pro)renin-synthesizing tissues under certain pathologic conditions. The (P)RR is highly expressed in the intercalated cells of the collecting duct in the kidney, where prorenin synthesis in the principal cells is increased in diabetic mellitus. Intercalated cells are important in regulating acid-base homeostasis, and are located adjacent to principal cells. Our in vitro study shows that prorenin stimulates V-ATPase activity via the (P)RR in intercalated cells. Thus, the collecting duct represents an interesting place where local prorenin levels may be high enough for interaction with the (P)RR to regulate V-ATPase activity. Homologous studies have revealed that the C-terminal fragment of the (P)RR is an accessory protein of V-ATPase that is conserved in all metazoans, and that the (pro)renin-binding domain has been acquired later during evolution, thus being present in vertebrates only [46]. Here we found that the (P)RR is important for V-ATPase regulation, independent of prorenin. Furthermore, (P)RR deletion is lethal in animal models even when limited to specific cells, in contrast to RAS component knockout [12-14]. Several studies have revealed that the (P)RR is important for V-ATPase integrity and Wnt signaling by acting as an adaptor between LRP6 and V-ATPase [11]. These findings illustrate the importance of the prorenin-independent functions of the (P)RR.

We used an unbiased proteomics approach to identify (P)RR-interacting proteins to better understand the prorenin-independent function(s) of the (P)RR (**Chapter 5**). Sortilin-1 was identified in this approach as a (P)RR-interacting protein, and the interaction was confirmed by immunoprecipitation. Interestingly, we found that the (P)RR post-transcriptionally controls sortilin-1 protein abundance, indicating that the (P)RR affects sortilin-1-regulated cellular processes. Sortilin-1 is a sorting protein playing an important role in regulating neuron viability and function. Since the (P)RR is abundantly expressed in the brain, while (P)RR gene mutations have been linked to X-linked mental retardation, epilepsy and X-linked Parkinsonism with spasticity (XPDS) [47, 48], one may speculate that the (P)RR, in interaction with other regulators, such as sortilin-1, determines neuron function. Interestingly, adenovirus-induced (P)RR knockdown in the brain, unlike (P)RR deletion in other cells, is not lethal and attenuates angiotensin II-dependent hypertension [49]. This, together with our findings on (P)RR-controlled renin release, suggest that the (P)RR also regulates the RAS independent of (pro)renin and that it may play a role in neuron function. Finally, it is worth to notice that sortilin-1 was recently identified as a regulator of LDL metabolism and ApoB secretion [41]. Moreover, genetic variance in the SORT1 gene associates with the LDL levels in plasma, and is a risk factor for cardiovascular diseases [39, 40]. Our study unexpectedly provides a potential molecular link between the co-

occurrence of hypertension and hypercholesterolemia. (P)RR may directly affect LDL clearance via the LDLR by affecting sortilin-1 protein stability. Moreover, given its interaction with LRP6 [11], which is a homologue of the LDL clearance receptor LRP1, there is additional reason to believe that the (P)RR plays a role in LDL metabolism. Thus, we propose that (P)RR interacts with LDL receptors, including LDLR, LRP1, sortilin-1, and SORLA1 (sorting protein-related receptor), thereby controlling LDL metabolism. It is already known that dissociation of LDL from the LDLR requires a low pH (≈4). After dissociation, the LDLR is recycled and targeted to the membrane, while LDL is targeted to the lysosomes for degradation. Failure in ligand and receptor dissociation will target the complex to the lysosomes for degradation. This is where the (P)RR may come into play: it might regulate LDL metabolism by controlling LDL receptor recycling. If true, (P)RR deletion would decrease LDL uptake and suppress the expression of LDLR and sortilin-1 due to impaired receptor recycling. Reversely, when overexpressing the (P)RR, there will be increased LDL uptake. (P)RR gene polymorphisms may therefore not only associate with hypertension [50] but also with lipid disorders. Now that the measurement of soluble (P)RR in blood plasma is feasible [51], the soluble (P)RR levels might even be used as a biomarker in these diseases.

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Nederlandse samenvatting

De functie van prorenine, de voorloper van renine, bleef onbekend tot de (pro) renine receptor [(P)RR] ontdekt werd. (Pro)renine, indien gebonden aan de (P)RR, leidt tot de aanmaak van angiotensine en activeert intracellulaire signaaltransductie cascades zowel op een angiotensine II–afhankelijke als–onafhankelijke manier. Het concept dat (pro)renine optreedt als een (P)RR agonist die angiotensine-onafhankelijke signaaltransductie veroorzaakt, staat in het middelpunt van de belangstelling. Data verkregen uit transgene dieren met sterk verhoogde prorenine concentraties suggereren echter dat de effecten van prorenine afhankelijk zijn van angiotensine II vorming, terwijl transgene dieren met (P)RR overexpressie geen veranderingen laten zien in de concentraties van hun renine-angiotensine systeem (RAS) componenten. Hoewel in vitro studies inderdaad (pro)renine-geïnduceerde intracellulaire signaaltransductie via de (P)RR ondersteunen, moet worden opgemerkt dat de (pro)renine concentraties die in deze studies werden toegepast in de nanomolair range lagen, wat vele ordes van grootte hoger is dan de fysiologische (pro)renine niveaus. Zelfs onder pathologische omstandigheden, bijvoorbeeld in diabetes mellitus, zijn de prorenine concentraties in bloed hoogstens 5 tot 10 keer verhoogd, hetgeen nog steeds veel te weinig is voor directe, (P)RR-gemedieerde signaaltransductie. Bovendien is vastgesteld dat de (P)RR onderdeel is van het V-ATPase complex, wat onder andere een belangrijke rol speelt in de Wnt/β-catenine signaaltransductie cascade, onafhankelijk van (pro)renine. Het is daarom duidelijk dat de (P)RR ook belangrijke functies heeft die volledig los staan van het RAS. (P)RR knockout, in tegenstelling tot RAS component knockout diermodellen, is dodelijk, zelfs wanneer het gelimiteerd is tot 1 celtype, bijvoorbeeld de cardiomyocyt of de podocyt. Daarom blijft het fysiologische belang van (pro)renine-(P)RR interactie onduidelijk en dient het nader geanalyseerd te worden. Dit proefschrift heeft (pro)renine-(P)RR interactie en de (pro)renine-onafhankelijke (P)RR functie(s) nader onderzocht.

In Hoofdstuk 2 bestudeerden we in vitro de (pro)renine-(P)RR interactie met behulp van gladde spiercellen uit de vaatwand van de rat (VSMCs) die de humane (h) (P)RR tot overexpressie gebracht hadden. In combinatie met recombinant humane prorenine, stelde dit ons in staat om te bestuderen of (pro)renine-(P) RR interactie voorkomt. We vonden dat VSMCs met h(P)RR expressie, wanneer zij geïncubeerd werden met humaan prorenine, een aanzienlijk grotere hoeveelheid humaan prorenine bonden in vergelijking met wildtype VSMCs, en dit effect werd tenietgedaan door de h(P)RR uit te schakelen middels siRNA. Dit is een belangrijk bewijsstuk dat prorenine zich inderdaad aan de (P)RR bindt, tenminste in de geteste concentratie (80 nM). Bovendien stimuleerde 20 nM prorenine Erk1/2 fosforylering in VSMCs met h(P)RR expressie, en werd dit effect eveneens tenietgedaan door de h(P)RR uit te schakelen. Aangezien VSMCs geen angiotensinogeen kunnen maken kan de gevonden Erk1/2 activatie niet veroorzaakt zijn door angiotensine II vorming. Toch is de prorenine concentratie benodigd voor directe (P)RR signaaltransductie vele malen hoger dan de normale plasma waardes in vivo. Zulke prorenine waarden

komen waarschijnlijk niet voor in vivo in non-(pro)renine vormende weefsels.

De (pro)renine concentraties die benodigd zijn voor (pro)renine-(P)RR interactie en directe (P)RR-gemedieerde signaaltransductie kunnen verschillend zijn in verschillende celtypes. Advani et al. lieten zien dat in renale verzamelbuis cellen, zowel renine als prorenine in staat zijn om Erk1/2 fosforylatie te stimuleren, bij concentraties vanaf 20 pM. Deze concentratie is ongeveer 4 maal hoger voor prorenine en 40 maal hoger voor renine dan die bij de mens (respectievelijk ~5 pM en ~0.5 pM). Het ligt voor de hand dat de door (pro)renine gestimuleerde Erk1/2 activatie in verzamelbuis cellen V-ATPase activiteit vereist. Inderdaad leidde blokkade van V-ATPase activiteit door bafilomycine A1 tot een volledige blokkade van de (pro)renine-geïnduceerde Erk1/2 activatie. Bovendien is aangetoond dat de (P)RR co-lokaliseert met V-ATPase in verzamelbuis cellen. Alles bij elkaar genomen suggereert dit dat V-ATPase de (P)RR signalering faciliteert en bestaat de mogelijkheid dat in cellen waarin (P) RR co-lokaliseert met V-ATPase op het celoppervlak, kleinere hoeveelheden (pro) renine nodig zijn voor angiotensine-onafhankelijke (P)RR signaaltransductie.

V-ATPases komen in vrijwel alle celtypen tot expressie in intracellulaire compartimenten. In speciale cellen, zoals de intercalated cellen van de verzamelbuis, is er ook V-ATPase expressie op het celoppervlak. Het is interessant dat de (P) RR in deze cellen co-lokaliseert met V-ATPase. Daarom is de verzamelbuis een voor de hand liggende plaats voor (P)RR-V-ATPase interactie waar relatief kleine hoeveelheden (pro)renine voldoende zouden kunnnen zijn om signaaltransductie via de (P)RR te initiëren. Ook is zowel de (P)RR expressie als de produktie van prorenine verhoogd in de verzamelbuis bij patiënten met diabetes mellitus, wat er mogelijk op wijst dat onder zekere (diabetische) omstandigheden er inderdaad lokaal voldoende hoge prorenine concentraties bereikt worden om te binden aan de (P)RR. Wel blijft het de vraag of prorenine-(P)RR interactie de V-ATPase activiteit direct beïnvloedt. Daarom hebben we de rol van de (P)RR in V-ATPase regulatie bestudeerd.

In Hoofdstuk 4 vonden we dat prorenine dosis-afhankelijk de V-ATPase activiteit in MDCK.C11 cellen stimuleert. Deze cellen lijken op de intercalated cellen van de verzamelbuis. In de geteste range (10 pM tot 10 nM), was er een duidelijke stimulatie van V-ATPase activiteit door prorenine bij 1 nM en 10 nM. Het effect van prorenine op V-ATPase was onafhankelijk van angiotensine II vorming, daar de angiotensine II type 1 en 2 (AT1, AT2) receptor blokkers irbesartan en PD123319 geen verandering teweegbrachten in de door prorenine gestimuleerde V-ATPase activiteit. Verder leidde de uitschakeling van de (P)RR middels siRNA tot een complete blokkade van door prorenine gestimuleerde V-ATPase activiteit, wat de indruk wekt dat het effect inderdaad mede door de (P)RR veroorzaakt wordt. De benodigde prorenine concentratie echter, hoewel 20 keer lager dan die voor de stimulatie van Erk1/2 in VSMCs, is nog steeds meer dan 100 keer hoger dan de hoeveelheid prorenine die bij mensen voorkomt. Ondanks de vraag of deze concentratie al dan niet behaald kan worden op het niveau van de verzamelbuis, is het belangrijk om de fysiologische

relevantie van (pro)renine-(P)RR interactie voor V-ATPase regulering te valideren.

Er zijn twee belangrijke (pro)renine bronnen in de nier: de juxtaglomerulaire cellen en de principal cellen van de verzamelbuis [18]. Verhoogde prorenine waarden in plasma correleren sterk met de progressie van diabetes. Tevens lijkt een diabetisch model in ratten erop te wijzen dat de verzamelbuis de grootste bron voor de verhoogde hoeveelheden prorenine is in deze ziekte [19]. Het is echter nog niet duidelijk of de grotere hoeveelheid prorenine in patiënten met diabetes mellitus uit de verzamelbuis afkomstig is. Daarom hebben we in Hoofdstuk 3 de hoeveelheid renine en prorenine in urine gemeten in patiënten met type 2 diabetes (T2D) na een 2 maanden durende behandeling met placebo, de renine remmer aliskiren, de AT1 receptorblokker irbesartan of aliskiren + irbesartan. Alhoewel T2D patiënten verhoogde plasma waarden van prorenine hadden, konden we geen prorenine in de urine monsters vinden. Na behandeling met aliskiren, irbesartan, of een combinatie van beide, waren de renine waarden in plasma 2.5 tot 10 maal hoger in vergelijking met de placebo behandeling, terwijl de renine waarden in urine onveranderd bleven. Dit leidt tot lagere urine/plasma verhoudingen voor de renine concentraties. Onze studie suggereert twee mogelijkheden ten aanzien van de locatie(s) waar renine in urine vandaan kan komen. De eerste optie is dat het uit een bron komt die onafhankelijk van de juxtaglomerulaire cellen gereguleerd wordt. De tweede optie is dat de renine in urine zowel uit deze bron als uit het bloed komt. Ondanks de mogelijkheid dat de verzamelbuis mogelijk (pro) renine aanmaakt onder pathologische omstandigheden, zien we dus geen prorenine in urine en zijn de renine concentraties in de urine ver beneden de range die nodig is om de (P)RR te stimuleren. Hier moet echter opgemerkt worden dat de locatie van de (pro)renine productie in de verzamelbuis de principal cellen betreft. Daarom is het nog steeds mogelijk dat de lokale hoeveelheden (pro)renine (in de buurt van de principal cellen) hoog genoeg zijn om de V-ATPase activiteit op een paracriene wijze te reguleren.

Ondanks de controverse rond (pro)renine-(P)RR interactie, wordt vermoed dat de (P)RR zelf (onafhankelijk van (pro)renine) een rol speelt in cardiovasculaire complicaties. De (P)RR expressie is verhoogd onder verscheidene pathologische condities, bijvoorbeeld in het hart en de nier van ratten met hartfalen, in het hart van spontaan hypertensieve ratten die gevoelig zijn voor een beroerte, in nieren van ratten met diabetische nefropathie, in de resterende nier van ratten na de verwijdering van 1 nier, en in de clipped nier van Goldblatt hypertensieve ratten. Bovendien resulteerde overexpressie van de (P)RR, zonder verandering van de waarden van de RAS componenten, in een verhoogde bloeddruk, verhoogde aldosteron concentraties in bloed, verhoogde cyclooxygenase-2 expressie in de nier, en glomerulosclerosis. Blokkade van de (P)RR kan daarom positieve effecten hebben ten aanzien van het voorkomen of vertragen van cardiovasculaire complicaties. Er is een antagonist ontworpen op basis van het gedeelte van prorenine dat bindt aan de (P)RR, de zgn. handle region ('handle region peptide', HRP). Alhoewel er gunstige effecten van HRP gerapporteerd zijn in diermodellen, suggereren diverse studies ook dat HRP als een gedeeltelijke agonist van de (P)RR zou kunnen fungeren, of dat het mogelijk (P)RR-onafhankelijke effecten heeft.

We hebben daarom de effecten van de HRP op (pro)renine-(P)RR interactie onderzocht. In Hoofdstuk 2 vonden we dat HRP geen invloed heeft op de DNA synthese die veroorzaakt wordt door prorenine + angiotensinogeen in VSMCs, wat erop wijst dat het geen invloed heeft op de prorenine activatie door (P)RR. Bovendien veranderde 1 μM HRP niet de (pro)renine-geïnduceerde Erk1/2 activatie. Dit wijst erop dat HRP geen blokkerend effect heeft op zowel de angiotensine-afhankelijke als de –onafhankelijke (P)RR signaaltransductie. In Hoofdstuk 4 hebben we de effecten van HRP op (pro) renine-(P)RR interactie in relatie tot V-ATPase regulering verder onderzocht. Helaas blokkeerde 1 μM HRP niet de door 10 nM prorenine-geïnduceerde V-ATPase activiteit in MDCK.C11 cellen. Opvallend genoeg stimuleerde HRP juist zelf de V-ATPase activiteit, op een dosis-afhankelijke wijze. (P)RR uitschakeling veroorzaakte een complete blokkade van de effecten van prorenine en HRP, wat suggereert dat beiden V-ATPase (P)RR-afhankelijk stimuleren. Onze data laten zodoende duidelijk zien dat HRP inderdaad een functie kan hebben als agonist. Dit is echter in tegenspraak met eerdere bevindingen dat HRP positieve invloed heeft op eind-orgaanschade in ziektemodellen door de (P)RR te blokkeren. De discrepantie tussen de diverse studies blijft moeilijk te verklaren, vooral doordat de functie(s) van (P)RR nog niet volledig ontrafeld zijn.

De afgelopen paar jaar is naar voren gekomen dat (P)RR een belangrijke rol speelt in Wnt signalering en ten aanzien van de V-ATPase samenstelling, onafhankelijk van (pro)renine. In Hoofdstuk 4 vonden we dat uitschakeling van de (P)RR geen invloed heeft op de basale V-ATPase activiteit, maar wel de door vasopressine gestimuleerde V-ATPase activiteit onderdrukte, wat aantoont dat de (P) RR V-ATPase ook onafhankelijk van prorenine reguleert. We vonden bovendien dat uitschakeling van de (P)RR niet tot een verminderde werking van alle Vo subunits leidt, zoals beschreven in voorgaande studies, maar tot een selectieve verlaging van Voa2. In de nier is Voa4 de meest voorkomende en dominante Voa subunit van V-ATPase. Deze subunit komt voornamelijk tot expressie op de plasmamembraan. Deze data verklaren waarom de basale V-ATPase activiteit intact bleef na uitschakeling van de (P)RR, terwijl stimulatie niet meer mogelijk was. Ze benadrukken tevens nogmaals de prorenine-onafhankelijke functie van de (P)RR bij V-ATPase regulering. Hoewel de (P)RR dus belangrijk is voor de samenstelling en regulering van V-ATPase, is er weinig bekend over de gevolgen van (P)RR-V-ATPase interactie in de cel. Recentelijk vonden Cruciat et al. dat de (P)RR als adaptor optreedt tussen de Frizzled coreceptor LRP6 en V-ATPase en onmisbaar is voor Wnt signaaltransductie [11]. Dit suggereert dat de (P)RR als link tussen V-ATPase en andere receptoren/ eiwitten zou kunnen fungeren, waarbij het verschillende celprocessen reguleert. In Hoofdstuk 5 gebruikten we proteomics om eiwitten die een directe interactie vertonen met (P)RR hypothesevrij te identificeren. (P)RR met een N-terminaal TAP tag kon worden opgezuiverd uit HEK293 cellen, en de eiwitten die hiermee interacteerden werden geïdentificeerd door middel van massaspectrometrie. Met deze aanpak vonden we, niet verrassend, dat verscheidene V-ATPase subunits gebonden aan de (P)RR voorkwamen, waaronder de eerder beschreven VoD en VoC subunits. Dit ondersteunt de effectiviteit van onze methode. Daarnaast vonden we een paar honderd

andere eiwitten die een wisselwerking met de (P)RR vertonen, waaronder eiwitten die betrokken zijn bij signaaltransductie, de afbraak van lipiden, en het in stand houden van de cytoskeletstructuur. Een van deze potentiële bindingspartners van de (P)RR was sortiline-1. We valideerden deze interactie met gebruik van immunoprecipitatie. We zagen bovendien dat sortilin-1 en (P)RR wederzijds elkaars eiwithoeveelheid reguleerden: verwijdering van de (P)RR leidde tot verminderde sortiline-1 expressie en omgekeerd. Verwijdering van de (P)RR had geen effect op de sortilin-1 mRNA expressie, noch had de verwijdering van sortilin-1 een effect op de (P)RR mRNA expressie, wat erop wijst dat het effect op eiwithoeveelheid na de transcriptie plaatsvindt.

Sortiline-1, ook bekend als neurotensine receptor 3, wordt gecodeerd door het SORT1 gen. Deze receptor bindt meerdere liganden. Sortiline-1 komt veel voor in neuronen, en regelt de intracellulaire routing van APP, de voorloper van amyloid-β (Aβ), de belangrijkste component van ouderdomsplaque in de hersenen van Alzheimer patiënten. Sortiline-1 bindt ook ApoE met hoge affiniteit en is verantwoordelijk voor de verwijdering van ApoE/Aβ uit neuronen. Dit wijst op een intrigerende rol van sortiline-1 bij de ziekte van Alzheimer. Recente GWA studies ondersteunen een verband tussen SORT1 en LDL-C. Inderdaad is sortiline-1 een klaringsreceptor voor plasma LDL. Het is dus mogelijk dat, door controle van de sortiline-1 concentratie, de (P)RR een onverwachte rol speelt bij diverse cellulaire processen. Dit kan wellicht ook verklaren waarom het leven zonder (P)RR niet mogelijk is, zelfs wanneer de (P)RR knockout maar beperkt is tot enkele celtypes.

Directe renine remmers vormen een nieuwe klasse van antihypertensieve medicatie die gericht is op de blokkade van het RAS. Aliskiren is het eerste medicijn in deze klasse dat is goedgekeurd voor klinisch gebruik. Het verlaagt op dosis-afhankelijke wijze de bloeddruk. Recent is er een nieuwe directe renine remmer ontwikkeld: VTP-27999. Deze stof heeft een vergelijkbare IC50 en halfwaardetijd als aliskiren, maar een bijna 10 keer hogere biologische beschikbaarheid. We vergeleken deze remmer met aliskiren in Hoofdstukken 6 en 7. Net als aliskiren liet VTP-27999 een acute verhoging, tot het 20-voudige, van de renine concentraties zien, en verhoogde het renine zelfs >100-voudig na 10 dagen behandeling. Plasma renine daalde weer nadat met de behandeling gestopt werd, met een halfwaardetijd van 39±10 uur in de met aliskiren behandelde gezonde vrijwilligers en 30±3 uur bij de met VTP-27999 behandelde gezonde vrijwilligers, wat in lijn ligt met de halfwaardetijd van deze twee remmers. Naast binding aan renine kan aliskiren ook aan prorenine binden. Dit leidt tot activatie van prorenine zonder dat het zgn. prosegment wordt verwijderd (i.e., niet-proteolytisch), hetgeen vervolgens de meting van prorenine in een immunoradiometrische assay (IRMA) voor renine mogelijk maakt. Onverwacht vonden we dat VTP-27999 aan prorenine bond zonder de 3D structuur van prorenine te veranderen, i.e., er was geen activatie. Wel kon VTP-27999, net als aliskiren, zuur-geactiveerd prorenine in zijn open conformatie vergrendelen. We maakten ook de verrassende observatie dat VTP-27999-binding een toename in renine immunoreactiviteit veroorzaakte, zodat er meer renine werd gemeten in de renine IRMA. Dit gebeurde niet bij aliskiren. Op basis van de verschillen tussen VTP-27999 en aliskiren

is het mogelijk dat dat beide remmers verschillen in weefselpenetratie/intracellulaire ophoping alsmede ten aanzien van interferentie met de (pro)renine-(P)RR interactie.

Daarom bestudeerden we de intracellulaire ophoping van aliskiren en VTP-27999 in renine-producerende humane mestcellen (HMC-1). We vonden dat VTP-27999 zich ophoopt in HMC-1 cellen met ≈5 maal hogere concentraties dan aliskiren. Dergelijke verschillen in ophoping werden ook gezien bij non-renine-producerende HEK293 cellen, wat suggereert dat de aliskiren en VTP-27999 ophoping renine-onafhankelijk is. Onze bevindingen, tenminste in het geval van aliskiren, zijn in lijn met een eerdere studie die aantoonde dat aliskiren ophoping in de nier niet gerelateerd is aan (pro)renine. Met gebruik van fluorescerend gelabeld aliskiren zagen we een bescheiden verrijking van aliskiren in mitochondriën en lysosomen, onafhankelijk van renine. Zoals hieroven vermeld, verandert VTP-27999 de renine immunoreactiviteit die gedetecteerd wordt door active site-gerichte antilichamen. Deze wijziging wordt waarschijnlijk veroorzaakt door veranderingen in de renine conformatie na binding aan VTP-27999. Zulke veranderingen kunnen in theorie ook invloed hebben op de klaring van renine via mannose 6-fosfaat receptoren (M6PR). We vonden dat in aanwezigheid van VTP-27999 de (pro)renine ophoping in VSMCs een ≈14-voudige verhoging liet zien in vergelijking met (pro)renine opname zonder VTP-27999. M6P blokkeerde de renine/prorenine ophoping in VSMCs zowel met als zonder VTP-27999, hetgeen suggereert dat dit proces M6PR-afhankelijk is en niet beïnvloed wordt door VTP-27999 binding. Wel lijkt de intracellulaire afbraak van VTP-27999-gebonden renine trager te verlopen dan de afbraak van vrij renine, zodat kennelijk VTP-27999 binding, net als aliskiren binding, zorgt voor stabilisatie van renine. Tenslotte bestudeerden we of VTP-27999 binding invloed had op de (pro)renine-(P)RR interactie. Zowel renine als prorenine veroorzaakten Erk1/2 fosforylatie in VSMCs. Aliskiren had hier geen invloed op, zoals verwacht, maar VTP-27999 blokkeerde de effecten van renine, doch niet van prorenine. Dit bevestigt dat VTP-27999 inderdaad invloed kan hebben op de renine conformatie, daarmee de interactie tussen renine en de (P)RR veranderend.

De (P)RR vertoont een wisselwerking met V-ATPase en speelt een belangrijke rol bij de regulering van V-ATPase activiteit. De door V-ATPase gemedieerde verzuring van intracellulaire compartimenten kan o.a. de omzetting prorenine naar renine beïnvloeden. Daarom bestudeerden we het effect van (P)RR uitschakeling op de (pro) renine afgifte door HMC-1 cellen. We vonden dat de basale afgifte van renine, en in mindere mate van prorenine, toenam bij uitschakeling van de (P)RR met gebruik van siRNA, op een tijdsafhankelijke wijze. Dit effect hing samen met een initiële afname van het renine dat in de cellen lag opgeslagen, en een verhoogde renine mRNA waarde, 48 uur na uitschakeling van (P)RR. Deze verhoogde renine expressie resulteerde erin dat de cellulaire renine waarden na 72 uur terug waren op het normale niveau, en dat de renine mRNA waarden op 96 uur na (P)RR uitschakeling weer terug waren op het normale niveau. Kennelijk leidt uitschakeling van de (P)RR dus tot een acute afgifte van opgeslagen renine, die vervolgens gecompenseerd wordt door een verhoogde renine expressie zodat uiteindelijk de renine opslag in de cel gelijk blijft.

(P)RR uitschakeling beïnvloedde de door forskoline of db-cAMP geïnduceerde renine afgifte niet. Samenvattend laat onze studie zien dat de (P)RR de prorenine-renine omzetting bepaalt en de basale (maar niet de gestimuleerde) renine afgifte regelt.

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Curriculum Vitae

Xifeng Lu was born on the 11th of December 1984 in Baiyin, Gansu province in the northwest of China. He received his first degree (BSc) of Biotechnology at China Agricultural University in Beijing in 2007. Then he came to The Netherland to receive further training in Biotechnology in Wageningen, and received his Master degree in 2009. Thereafter, he started his PhD research at the Division of Vascular Medicine and Pharmacology, of the Department Internal Medicine, at Erasmus Medical Center, Rotterdam. This research focused on the function of the (Pro)renin Receptor and Renin-Angiotensin-System. The results of this research are presented in this thesis.

Publications

Lu X, Danser AH, and Meima ME. HRP and prorenin: focus on the (pro)renin receptor and vacuolar H+-ATPase. Frontiers in bioscience 3: 1205-1215, 2011.

Batenburg WW, **Lu X,** Leijten F, Maschke U, Muller DN, and Danser AH. Renin- and prorenin-induced effects in rat vascular smooth muscle cells overexpressing the human (pro)renin receptor: does (pro)renin-(pro)renin receptor interaction actually occur? Hypertension 58: 1111-1119, 2011.

Krop M, **Lu X**, Danser AH, and Meima ME. The (pro)renin receptor. A decade of research: what have we learned? Pflugers Archiv : European journal of physiology 465: 87-97, 2013.

Krop M, **Lu X**, Verdonk K, Schalekamp MA, van Gool JM, McKeever BM, Gregg R, and Danser AH. New Renin Inhibitor VTP-27999 Alters Renin Immunoreactivity and Does Not Unfold Prorenin. Hypertension 61: 1075-1082, 2013.

Lu X, Garrelds IM, Wagner CA, Danser AH, Meima ME. (Pro)renin Receptor is Required for Prorenin-Dependent and -Independent Regulation of Vacuolar H+-ATPase Activity in MDCK.C11 Collecting Duct Cells. Am J Physiol Renal Physiol. In Press.

Persson F*, **Lu X***, Rossing P, Garrelds IM, Danser AH, Parving HH. Urinary renin and angiotensinogen in type 2 diabetes: added value beyond urinary albumin? J Hypertens. 2013 Jun 5. In Press. (*: Both author contributed equally.)

Lu X, Roksnoer LCW and Danser AH. The Intrarenal Renin-angiotensin System: Does It Exist? Implications from a Recent Study in Renal ACE Knockout Mice. Nephrol Dial Transplant. In Press.

Conference Presentations

High Blood Pressure Research Conference, New Orleans, LA, U.S, September 2013. "The (Pro)renin Receptor Regulates Renin Release and Prorenin-to-Renin Coversion in (Pro)renin-Synthesizing Human Mast Cells" (Poster Presentation)

High Blood Pressure Research Conference, Orlando, FL, U.S, September 2011. "Prorenin Activates Vacuolar H+-ATPase via (Pro)renin Receptor in Collecting Duct Cells" (Oral Presentation)

Benelux Congress on Physiology and Pharmacology (Physphar), Liege, Belgium, March 2011. "The (Pro)renin Receptor Is Required for Vacuolar H+-ATPase Regulation in Collecting Duct Cells" (Poster Presentation)

High Blood Pressure Research Conference, Washington DC, U.S, September 2010. "The (Pro)renin Receptor Is Required for Vacuolar H+-ATPase Regulation in Collecting Duct Cells" (Poster Presentation)

Awards and Grants

New Investigator Award Travel Grant- HBPR	2011
ISH Presentation Prize - HBPR	2011
NSFW Travel Grant	2011

PhD Portfolio

Name PhD student:	Xifeng Lu
Erasmus MC Department:	Department of Internal Medicine Division of Vascular Medicine and Pharmachology
Research School:	COEUR
Promotor:	Prof.dr. A.H.J. Danser
Copromotor:	Dr. Marcel E. Meima

PhD training

	Year	ECT
Akademic skills, teaching and courses		
Extracellular Matrix Course	2010	0.4
Confocal Introduction Course	2010	0.4
1 day Teaching Autonomic Nervous System	2010	0.3
Molecular Biology in Cardiovascular Research	2011	1.5
Heart Failure Research	2011	1.5
NHS Course Cardiac Function and Adaptation, Papendal	2011	2.0
Biomedical English Writing and Communication	2012	2.0
1 day Teaching Autonomic Nervous System	2012	0.3
NHS Course Vascular Biology, Papendal	2012	2.0
1 day Teaching Autonomic Nervous System	2013	0.3
Training at Noam Zelcer's lab (AMC, Amsterdam)	2013	4.4
Genetics for Dummies	2013	0.5
Workshop Successful Grant Proposal Writing	2013	0.5
Cardiovascular Medicine	2013	1.5
Presentations		
(Pro)Renin Receptor Meeting, Paris	2010	0.8
PhysPhar Meeting, Liege	2011	0.6
(Pro)Renin Receptor Meeting, Paris	2011	0.8
TI Pharma Meeting, Basel	2011	0.8
Council for High Blood Pressure Research, Orlando	2011	1.7
German Hypertension Society, Berlin	2012	0.8
TI Pharma Meeting, Rotterdam	2012	0.8
Presentations at the Division of Pharmacology	2010-2013	5.6
Conferences, seminars and symposia		
Cell Signaling Techniques Symposium, Leiden	2010	0.3
Science Days Internal Medicine, Antwerp	2010	0.6
Dutch Society of Pharmacology Spring Meeting, Rotterdam	2012	0.8
COEUR Research Seminar The Cardiovascular System	2012	0.4
COEUR Research Seminar Glucose Metabolism	2012	0.4
Erasmus/COEUR Lectures	2010-2013	0.9