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. (Hypertension. 2011;58:00-00.)

Key Words: prorenin ■ DNA synthesis ■ transgenic ■ (pro)renin receptor ■ renin ■ plasminogen activator inhibitor 1 ■ ERK1/2

Since the discovery of the (pro)renin receptor ([P]RR), 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.3,4 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 proteins, such as Wnt1, Wnt5a, Wnt10b, and Wnt11, in mediating autophagy-dependent survival and cell death.14,15 The (P)RR has been found to be upregulated in human atherosclerotic plaques,16 indicating that it may play a role in atherogenesis.

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molecules.\textsuperscript{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 $\approx 1$ to 20 nmol/L depending on the use of immobilized receptors or membrane fractions of (P)RR-transfected cells.\textsuperscript{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 ($\approx 0.5$ pmol/L) and prorenin ($\approx 5.0$ pmol/L) in extracellular fluid\textsuperscript{17} but explain why, in most in vitro studies, investigating (pro)renin-(P)RR interaction high nanomolar ($\approx 100$ nmol/L)\textsuperscript{10} (pro)renin concentrations were required to observe effects. 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.\textsuperscript{18} This handle region peptide (HPP) 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,\textsuperscript{19,20} clear evidence that this peptide blocks prorenin-(P)RR interaction is lacking. It has even been claimed that HPP also prevents renin binding to the (P)RR, acts as an agonist of the (P)RR, and/or has (P)RR-independent effects.\textsuperscript{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 aortic vascular smooth muscle cells (VSMCs) and their wild-type controls.\textsuperscript{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 mannos 6-phosphate receptors (M6PRs). The latter receptors are clearance receptors for both renin and prorenin, but have also been linked to signaling.\textsuperscript{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)RR\textsuperscript{1} and their control littermates (n=8 for both).\textsuperscript{25} In short, VSMCs were isolated from the aorta, pooled, plated, and maintained at 37°C in a humidified 5% CO\textsubscript{2} incubator in supplemented SmBm-2 medium (Cambrex) containing 10% FBS. Cells were cultured to confluence in a 75-cm\textsuperscript{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 $\approx 4 \times 10^5$ 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'-GAGAUAUGCAGUUCCCUU-3', 5'-CAACCUUUGCGUAAUAGU-3', 5'-CAACAUUUGGAAUAGGAAU-3': human[P]RRsiRNA:5'-GGACUAUCCUGAGGGCAAAAA-3', 5'-GAGUGUUAUGGAGGAAA-3'; control siRNA: silenter 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 (Actelson, 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.\textsuperscript{26}

**Binding Studies With Recombinant Rat and Human Renin and Prorenin**

To study the consequence of transfection on the binding of human renin, 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 nmol/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 N\textsubscript{2} 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.\textsuperscript{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 antibody\textsuperscript{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 nmol/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 (RILJKKMPSV-OH, to block the rat [P]RR), 1 nmol/L to 1 µmol/L of human HRP (IFLKRKMPSI-OH, to block the human [P]RR; Biosyntan, Berlin, Germany), or 0.1 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, $^3$H-thymidine was added to a final concentration of 0.5 µCi/mL in each well. $^3$H-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 $\approx 15$ minutes. Next, the cell lysates were centrifuged at 14 000 g at 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 secondary antibody (goat antirabbit, 1:5000) was used to visualize the receptor. Total mRNA was isolated from TRZol-lysed cells with a combined protocol of QIAzol lysis reagent and Qiagen RNeasy minikit.
The effect of 4 nmol/L of prorenin on ERK1/2 and ERK1/2. Results were expressed as percentage of phosphorylated p42/44 mitogen-activated protein kinase (phosphorylated ERK1/2 and ERK1/2). 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). 

**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 siRNA (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, ²H-thymidine incorporation in wild-type and h(P)RR VSMCs after incubation with 4 nmol/L of prorenin ± 150 nmol/L of angiotensinogen (AngII) 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 ²H-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 ²H-thymidine incorporation in wild-type (E) and h(P)RR (F) cells (mean±SEM of n=6). *P<0.05 vs control, #P<0.05 vs wild-type.

**Extracellular Signal-Regulated Kinase 1/2**

To study the effects of renin and prorenin on ERK1/2 phosphorylation, cells were incubated at 37°C for 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, 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;
incubation with 1 nmol/L of renin (R) themselves.15,28 Rat HRP (n = 8) and 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.

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.

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 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).

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 23% versus 146% (169%)) in both cell types (n = 6–7). *P < 0.05 vs baseline.

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.

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.
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).

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.

**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.

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. 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% (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 and no direct prorenin effects.

In contrast to prorenin, human renin, at concentrations ranging from 1 to 20 nmol/L (ie, ~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, 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 before7,15 and raises the possibility that the beneficial effects of these drugs19,20 involve other, as-yet-undefined targets.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 (eg, 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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Wendy W. Batenburg, Xifeng Lu, Frank Leijten, Ulrike Maschke, Dominik N. Müller and A.H. Jan Danser

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