R enin inhibitors, like all renin-angiotensin system (RAS) blockers, increase the concentration of renin because they attenuate the negative feedback effect of angiotensin (Ang) II on renin release. Because this rise has been suggested to be larger than during other types of RAS blockade,1 it may limit the effectiveness of renin inhibitors, either because angiotensin generation will occur, or because such high renin levels will activate the (pro)renin receptor ((P)RR).2 Such activation results in mitogen-activated protein kinase (MAPK) activation.3 Several mechanisms could underlie the large rise in renin during renin inhibition. First, because the rise in renin reflects the degree of RAS blockade,4 it may point to highly efficient blockade. Second, it may be an artifact, because renin “activates” the precursor of renin, prorenin (Figure 1A).5 This is because of the fact that the prorenin prosegment, in a pH- and temperature-dependent manner, is capable of unfolding from the enzymatic cleft, thereby resulting in 2 prorenin conformations: a “closed,” inactive form, and an “open” form that displays enzymatic activity. Under physiological conditions, <2% of prorenin is in the open conformation. Renin inhibitors bind to prorenin in the open conformation, to the same degree as they bind to renin. However, because of the presence of the renin inhibitor, the inactivation step (ie, the return to the closed conformation) is now no longer possible, and thus, the equilibrium between the closed and open conformation will shift into the direction of the open conformation. Eventually, depending on time, temperature, the concentration of the inhibitor, and its affinity for the active site of renin, a significant proportion of prorenin may be open, allowing its recognition by the active site-specific antibodies used in renin assays, despite the fact that the prosegment is still present (“nonproteolytic activation”).6 This recognition of prorenin as renin may particularly contribute to the overestimation of renin,6 because prorenin levels are >10-fold higher than renin levels. Finally, as suggested by Sealey and Laragh,1 the rise in renin during aliskiren treatment may be attributable to interference with renin clearance. Renin clearance largely depends on its glycosylation pattern, because the carbohydrate moieties determine its uptake by Kupffer cells in the liver.7 The responsible clearance receptors are, among others, mannose 6-phosphate receptors (M6PRs).8–10 These receptors

Key Words: prorenin ■ renin ■ aliskiren ■ half-life ■ receptor
bind and internalize both renin and prorenin, and the 2 enzymes are subsequently degraded intracellularly with a half life of several hours.

In the present study, we set out to first establish whether aliskiren is capable of “activating” prorenin to the same degree as the first renin inhibitor that was used to demonstrate this phenomenon, remikiren.5 Secondly, we studied whether aliskiren affects renin/prorenin binding to the M6PR and (P)RR, making use of the M6PR blocker M6P,11 the (P)RR blocker HRP,12 and rat vascular smooth muscle cells (VSMCs) from transgenic rats with smooth muscle cell expression of the h(P)RR (h(P)RR).13 Third, we quantified prorenin clearance by these cells with and without aliskiren.

Methods

Collection of Blood Plasma
Blood for renin and prorenin measurements was collected from 14 subjects with renal artery stenosis (9 men, 5 women; ages 23 to 85 years) as described before.14 The samples were centrifuged at room temperature at 3000 g for 10 minutes, and plasma was stored at −20°C.

Cell Culture
All 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 smooth muscle cell expression of the h(P)RR and their control littermates.15 In short, VSMCs were isolated from the aorta, 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 cm2 flask (in

supplemented SmBm-2 medium; passage 3 to 8), trypsinized, and seeded into 24-well plates using the above medium. This yielded a confluent monolayer of ~4×10^6 cells per cm2 after 3 days. Before the start of the experiment the cells were cultured for 24 hours under serum-free conditions.

Prorenin/Renin Binding Studies
To distinguish cell surface-bound prorenin from intracellular prorenin, cells were loaded at 37°C with recombinant human prorenin (1000 U/L) in the absence or presence of M6P (10 mmol/L) for 2 hours. At the end of the incubation period, the cells were washed 3 times with 1 mL ice-cold PBS, and then either lysed immediately with ice-cold 0.2% triton X-100 in PBS or after a 10-minute incubation at 4°C with an acid-solution containing 50 mmol/L glycine and 150 mmol/L NaCl, pH 3.0, to remove cell surface-bound prorenin.11

To study the effect of renin inhibition, M6PR blockade or h(P)RR blockade on renin/prorenin binding, cells were incubated at 4°C or 37°C with prorenin (1000 U/L) or renin (600 U/L; prepared from prorenin as described before11) for up to 24 hours in the absence or presence of aliskiren (10 pmol/L to 10 μmol/L; a kind gift of Novartis Pharmaceuticals, Basel, Switzerland), remikiren (10 μmol/L), M6P (10 mmol/L) or HRP (IFLKRMPSI-OH, Biosyntan; 1 μmol/L).12 Next, the cells were washed and lysed as described above. For comparison, these experiments were also performed after permeabilization of the cells with PBS containing 0.2% saponin.8

To determine the half life of cellular prorenin, the cells were loaded with prorenin (1000 U/L) for 2 hours at 37°C, washed 3 times with 1 mL ice-cold PBS, and then either lysed immediately or after an additional incubation period of maximally 15 hours at 37°C in serum-free medium without renin or prorenin. Cell lysates and were stored at −70°C.

To verify intracellular prorenin metabolism in lysosomes, subcellular fractionation was performed15 using lysates of cells incubated with 1000 U/L prorenin for 24 hours at 37°C in the presence of 10 μmol/L aliskiren. In short, after the incubation, cells were washed 3 times with 1 mL ice-cold PBS and then lysed immediately in ice-cold Tris/sucrose buffer pH 7.4, containing 50 mmol/L Tris.HCl, 0.25 mol/L sucrose, 10 mmol/L EDTA, 3 mmol/L MgCl2, and 0.2% Triton X-100. The homogenate was centrifuged at 1000 g for 5 minutes to obtain the nuclear fraction. The supernatant was centrifuged again at 10 000 g for 10 minutes to obtain the mitochondrial fraction. The resulting supernatant was centrifuged at 20 000 g for 10 minutes to obtain the lysosomal fraction. Finally, the remaining supernatant was centrifuged at 180 000 g for 30 minutes to separate the microsomal fraction from the cytosol/extracellular fluid fraction. Pellets were resuspended in Tris/sucrose buffer and all fractions were stored at −20°C.

Angiotensin Generation and (P)RR Activation
To study whether aliskiren blocks prorenin-induced angiotensin generation, cells were incubated at 37°C with 150 mmol/L angiotensinogen and 1000 U/L prorenin. Medium samples (50 μL) were collected after 4 hours, mixed with 2.5 μL inhibitor stock solution,16 and stored at −70°C.

To study whether aliskiren blocks renin-induced (P)RR-mediated DNA synthesis, h(P)RR cells were incubated at 37°C with 30 U/L renin for 24 hours in the absence or presence of 10 μmol/L aliskiren. DNA synthesis rate was determined in duplicate by quantifying [3H]-thymidine incorporation during the last 6 hours of the above 24-hour incubation period.10

Biochemical Measurements
Protein was determined according to Bradford. Renin and prorenin were measured by a renin-specific immunoradiometric assay (IRMA; Renin III, CisBio). Prorenin-containing samples were incubated for up to 48 hours with aliskiren or remikiren (1 pmol/L to 10 μmol/L) at 4, 22, or 37°C to induce a conformational change in the prorenin molecule (“nonproteolytic activation”), which allows its recognition by the renin-specific monoclonal antibodies applied in the IRMA.15 To evaluate whether the activation was truly nonproteo-
prosegment-directed monoclonal antibody in the IRMA. Finally, to evaluate whether exposure to aliskiren or remikiren had induced complete activation of prorenin, prorenin was also determined by IRMA after its conversion to renin by trypsin (the “gold standard” of prorenin activation). Ang I and II levels in medium were measured by radioimmunoassay as described before.

In the fractionation studies, DNA, succinate dehydrogenase, acid phosphatase, NADPH cytochrome c reductase, and lactate dehydrogenase were measured as described before, to verify that the 5 fractions truly represented nuclei, mitochondria, lysosomes, microsomes, and cytosol, respectively.

Data Analysis
Results are expressed as mean±SEM, and n refers to the number of experiments. Statistical analysis was performed using 2-way ANOVA or t test. *P<0.05 was considered significant.

Results
Aliskiren-Induced Activation of Prorenin
Prorenin incubation with increasing concentrations of aliskiren at 4°C for 48 hours allowed its recognition in a renin-specific assay (Figure 1B, n=3). Maximum activation (as compared to the “gold standard”, i.e., trypsin activation) occurred when using 10 μmol/L aliskiren. Shorter incubations (24 hours) or incubations at higher temperatures (22°C or 37°C) did not yield better results (Figure 2A; n=2). When applying the optimal activation method (10 μmol/L aliskiren at 4°C for 48 hours) to human plasma samples obtained from 11 subjects with renal artery stenosis, the renin-specific assay yielded results that were identical to those obtained after trypsin activation (Figure 2B). Results with remikiren were indistinguishable from those with aliskiren (Figure 2B). Prorenin activation occurred nonproteolytically, because the levels of prosegment-containing prorenin in 3 subjects with renal artery stenosis (526±200 μU/mL) after the above optimal activation procedure were identical to the prorenin levels measured in the renin-specific assay (441±98 μU/mL). Thus, a 48-hour incubation at 4°C with 10 μmol/L aliskiren is sufficient to fully activate prorenin in a nonproteolytic manner.

Location of Cell-Associated Prorenin: Cell Surface Versus Intracellular
The level of cell-associated prorenin in wild-type and h(P)RR VSMCs after a 2-hour incubation with prorenin at 37°C was identical (n=3), and not affected by acid-wash (Figure 3). Neither prorenin nor renin could be detected in the acid-wash fluid. This indicates that the majority of cell-associated prorenin in both cell types in the absence of M6P was not located on the cell surface, but intracellular.

Coincubation with M6P reduced the cellular prorenin levels in wild-type and h(P)RR cells by 80±5% and 43±16% (n=3; Figure 3). This indicates that M6PRs contribute to prorenin binding in both cell types, and that their contribution is larger in wild-type cells. The remaining cell-associated prorenin (representing non–M6PR-bound prorenin) was ~2-fold higher in h(P)RR cells than in wild-type cells. Acid wash reduced the cellular prorenin levels in M6P-treated h(P)RR cells by ~50%, but did not affect prorenin binding in M6P-treated wild-type cells. Thus, during M6PR blockade, about half of the cell-associated prorenin was located on the cell surface in h(P)RR cells. Because the h(P)RR binds, but does not internalize prorenin, this prorenin most likely represents h(P)RR-bound prorenin. Cell surface binding was not apparent in wild-type cells during M6PR blockade.
Effect of Renin Inhibition on Binding and Uptake of Renin and Prorenin

When incubating wild-type and (P)RR cells with 1000 U/L prorenin at 37°C, the level of cell-associated prorenin increased in a time-dependent manner, reaching a maximum after 2 to 4 hours (Figure 4; n=3 for both). In the presence of 10 μmol/L aliskiren, a steady-state was reached after 12 to 18 hours, and the cell-associated levels under these conditions were up to 40-fold higher than without aliskiren (Figure 4). Already after 4 hours, a 2- to 3-fold difference in cell-associated prorenin levels was observed. On the basis of these data it was decided to apply a 4-hour incubation period in all further studies.

Figure 5A (n=12) shows that the increase in cell-associated prorenin levels was of similar magnitude when prorenin had been incubated with aliskiren for 48 hours at 4°C before its application to the cells without additional aliskiren application to the medium. Thus, the aliskiren-induced increase in cell-associated prorenin levels depends on the actual binding of aliskiren to the prorenin molecule. Cell-associated prorenin levels in the absence of aliskiren or M6P were 5.2±0.3 and 4.4±0.5 mU/mg protein, respectively.

Figure 5B (n=8) shows that the effect of aliskiren on renin uptake paralleled its effect on prorenin uptake. Cell-associated renin levels in the absence of aliskiren or M6P were 14.5±3.5 and 11.8±3.2 mU/mg protein, respectively.

The aliskiren-induced rise in cell-associated prorenin levels in saponin-pretreated cells amounted to 36±5% of level without inhibitors in the medium. *P<0.05 vs without inhibitor.

Figure 6 shows that the effects of aliskiren were more pronounced in h(P)RR cells in comparison to wild-type cells. Aliskiren (10 μmol/L) reduced these levels to 0.33±0.05 pmol/mL, versus 2.42±0.23 and 0.52±0.14 pmol/mL in h(P)RR cells. Aliskiren (10 μmol/L) reduced these levels to 0.33±0.05, 0.10±0.02, 0.45±0.03, and 0.09±0.01 pmol/mL, respectively (n=6 for all measurements; P<0.01).

Concentration-Dependency of the Aliskiren-Induced Effect

The aliskiren-induced increase in prorenin uptake occurred in a concentration-dependent manner (Figure 1C; n=3 for each concentration), and paralleled its effect on prorenin activation (Figure 1B).

Aliskiren Blocks Prorenin-Induced Angiotensin Generation but not Renin-Induced (P)RR Activation

After 4 hours of incubation, the Ang I and II levels in wild-type cells amounted to 1.79±0.53 and 0.51±0.17 pmol/mL, versus 2.42±0.23 and 0.52±0.14 pmol/mL in h(P)RR cells. Aliskiren (10 μmol/L) reduced these levels to 0.33±0.05, 0.10±0.02, 0.45±0.03, and 0.09±0.01 pmol/mL, respectively (n=6 for all measurements; P<0.01). Renin increased [3H]-thymidine incorporation in h(P)RR cells to...
Loading the cells with 1000 U/L prorenin resulted in cellular lysosomal prorenin accumulation. Half life of cell-associated prorenin with and without renin inhibition and evidence for lysosomal prorenin accumulation

Loading the cells with 1000 U/L prorenin resulted in cellular prorenin levels of 4.7 ± 0.1 and 3.0 ± 0.2 mU/mg protein in wild-type and h(P)RR VSMCs, respectively. Continued incubation of the cells after replacement of the incubation medium with new medium without prorenin resulted in a partial decrease in cell-associated prorenin levels (t1/2 2.9 hours, respectively; n = 6), and similar observations were made for prorenin in h(P)RR VSMCs (t1/2 2.6 and 1.4 hours; n = 9, P < 0.01 versus no inhibitor). Subcellular fractionation studies revealed that, relative to total protein, prorenin was enriched in lysosomes (Figure 6B; n = 5). Recoveries were > 80% (data not shown). The enrichment of the nuclear (DNA: 61 ± 12, 37 ± 6, 7 ± 6, 0 ± 0 and 9 ± 9 μg/mg protein), mitochondrial (sucinate dehydrogenase: 26 ± 3, 40 ± 7, 8 ± 2, 6 ± 1 and 0.1 ± 0.1 nmol/min/mg protein), lysosomal (acid phosphatase: 3.8 ± 0.4, 5.4 ± 0.7, 5.9 ± 1.1, 5.6 ± 0.9 and 3.2 ± 0.2 nmol/min/mg protein), microsomal (cytochrome c reductase: 300 ± 85, 401 ± 164, 458 ± 68, 1080 ± 556 and 548 ± 147 μmol/min/mg protein), and cytosolic (lactate dehydrogenase: 117 ± 37, 680 ± 138, 1155 ± 148, 634 ± 143 and 1328 ± 766 μmol/min/mg protein) marker in their respective fractions confirmed the identity of each fraction (n = 4 for all).

**Discussion**

This study shows that aliskiren, like remikiren, is capable of "activating" prorenin. Secondly, aliskiren and remikiren prolong the half life of M6PR-internalized renin and prorenin. No evidence was obtained for h(P)RR-mediated internalization of prorenin, nor did aliskiren affect prorenin binding to either the h(P)RR or the M6PR.

A 48-hour incubation at 4°C with 10 μmol/L aliskiren was sufficient to fully activate prorenin in a nonproteolytic manner. This conclusion is based on the observation that the amount of immunoreactive renin detected after this treatment equaled that measured after exposure to trypsin, the "gold-standard" of prorenin activation, whereas aliskiren-activated prorenin had not lost its prosegment. Incubation at higher temperatures for 24 or 48 hours did not result in complete prorenin activation. This relates to the fact that at these temperatures the equilibrium between the open and closed conformation of prorenin is shifted into the direction of the closed conformation (Figure 1A), not allowing aliskiren (or remikiren) to activate all prorenin molecules. The high concentrations of aliskiren required to fully activate prorenin are in agreement with earlier studies on remikiren (which has a comparable IC50 for renin) showing that renin inhibitor concentrations > 1 μmol/L are needed for optimal results.

The aliskiren concentrations in blood plasma during aliskiren treatment are 2 to 3 orders of magnitude lower than the concentration applied here. Yet, as shown in Figure 1B, nonproteolytic prorenin activation may already occur in vitro at these concentrations. To what degree this also occurs in vivo (at 37°C) is still being debated.

VSMCs, when incubated with prorenin at 37°C, bind and internalize the molecule, as evidenced by our inability to remove cell-associated prorenin by acid-wash after incubation at 37°C. Acid wash does remove prorenin when cells are incubated at 4°C, because at that temperature prorenin remains bound to cell surface receptors and does not internalize. M6P greatly reduced the cellular prorenin accumulation at 37°C, indicating that the majority of prorenin uptake is M6PR-mediated. Importantly, the M6P-induced reduction in prorenin binding was smaller in h(P)RR-overexpressing cells than in wild-type cells, in agreement with the concept that the former cells, in addition to the M6PR, also express the "activating" prorenin. Secondly, aliskiren and remikiren prorenin concentrations of aliskiren required to fully activate prorenin are in agreement with earlier studies on remikiren (which has a comparable IC50 for renin) showing that renin inhibitor concentrations > 1 μmol/L are needed for optimal results. The aliskiren concentrations in blood plasma during aliskiren treatment are 2 to 3 orders of magnitude lower than the concentration applied here. Yet, as shown in Figure 1B, nonproteolytic prorenin activation may already occur in vitro at these concentrations. To what degree this also occurs in vivo (at 37°C) is still being debated.

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Acid wash did not diminish the cellular prorenin levels in h(P)RR VSMCs after their incubation in the absence of M6P.
This most likely relates to the fact that h(P)RR-dependent prorenin binding was of much smaller magnitude than M6PR-dependent prorenin binding. Taken together, our data support M6PR-mediated binding and internalization of prorenin in wild-type and h(P)RR VSMCs, and h(P)RR-mediated binding of prorenin without internalization in h(P)RR VSMCs.

M6PRs continuously cycle between the cell surface and the intracellular compartment (Golgi and endosomes), and the majority of these receptors is located intracellularly.9,10 This is illustrated by the fact that prorenin binding at 4°C (when cycling does not occur) was 5 to 10 times lower than binding at 37°C. H(P)RR-binding of prorenin, through a conformational change, has been reported to result in nonproteolytic prorenin activation on the cell surface.2 This nonproteolytic activation of prorenin was not investigated in the present study. In fact, we deliberately measured cellular prorenin in all VSMC studies after treatment of the samples with 10 μmol/L aliskiren for 48 hours at 4°C. Therefore, this measurement represents “total” cellular prorenin: the sum of closed prorenin, open prorenin, and any prorenin that has been converted to renin. The reason for this approach is that during most incubations a renin inhibitor was present in the medium. Consequently, depending on the incubation time and temperature, prorenin would have reached various stages of activation, and its measurement without complete activation would not have yielded meaningful results.

Unexpectedly, coincubation with aliskiren at 37°C increased the cellular “total” (pro)renin levels of VSMCs after their exposure to renin or prorenin by up to 40-fold. Aliskiren did not alter cell surface-binding of prorenin at 4°C. This latter observation argues against the concept that aliskiren, by inducing a conformational change in the renin/prorenin molecule, affects their binding to M6PRs or h(P)RRs. The increased accumulation was also observed when using aliskiren-activated prorenin without the addition of aliskiren to the medium, or when performing the studies after permeabilization of the cells with saponin (allowing prorenin full access to all receptors, independently of receptor recycling and internalization). Taken together, these data indicate that the effect of aliskiren depends on its binding to (pro)renin, and involves a phenomenon beyond receptor binding and internalization. The almost complete prevention of the aliskiren-induced increase in renin/prorenin accumulation by M6P suggests that the M6PR is required for this phenomenon. The h(P)RR blocker HRP was without effect, and aliskiren did not affect renin/prorenin binding to h(P)RR cells in the presence of M6P. Because under these conditions binding is largely determined by the h(P)RR, these data support the idea that aliskiren does not affect renin/prorenin binding to the h(P)RR.

M6PRs are clearance receptors for renin and prorenin,9,10 and thus, because M6PR-binding per se is unaltered, the M6PR-dependent rise in cellular renin/prorenin levels most likely reflects a change in the clearance of internalized (pro)renin. Subcellular fractionation studies confirmed accumulation of prorenin at intracellular clearance sites, ie, the lysosomes. In both cardiac and endothelial cells renin and prorenin are degraded with a half life of several hours after their internalization via M6PRs.8,9 This was also the case in the wild-type and h(P)RR VSMCs in the present study. Aliskiren increased the half life of cell-associated prorenin in both cell types by a factor of 2 to 3, in support of a decrease in clearance. Remikiren induced an even larger increase in half life. In agreement with the increase in intracellular half life, the time required to obtain steady-state cell-associated (pro)renin levels in the presence of aliskiren increased from 4 to 12 hours (Figure 4).

Summarizing therefore, our data suggest that renin inhibitor binding to both renin and prorenin increases their stability, making these molecules more resistant against degrading enzymes after their internalization. It is already well-known that renin inhibitors alter the 3D structure of renin.19 The aliskiren effect occurred in a concentration-dependent manner, starting at concentrations in the low μmolar range (Figure 1C). Such concentrations do occur in blood during aliskiren treatment, and, assuming that this aliskiren-induced stabilization of renin and prorenin affects all clearance pathways of renin and prorenin in vivo, it is likely that this phenomenon contributes to the rise in renin after renin inhibition. The effect might be particularly apparent when combining renin inhibitors with other drugs that also stimulate renin, like diuretics, ACE inhibitors, and AT1 receptor antagonists.1

In conclusion, this study provides 2 aliskiren-induced mechanisms that could potentially contribute to the large rise in renin after aliskiren treatment: the detection of nonproteolytically activated prorenin as renin, and a decrease in renin/prorenin clearance. Future studies, involving assays that recognize the prosegment of prorenin,8 are required to determine to what degree immunoreactive renin represents true renin or nonproteolytically activated prorenin. It is unlikely that the large increases in renin or prorenin will result in angiotensin generation, because they depend on aliskiren binding to the active site. In fact, aliskiren blocked prorenin-induced angiotensin generation in the present study by >80%. It is possible however that the increased renin/prorenin levels activate the h(P)RR, because aliskiren did not prevent such binding, nor did it block the renin-induced DNA synthesis in h(P)RR cells. The consequences of such h(P)RR overstimulation in humans are currently unknown, and should be evaluated in the light of the recent observation that excessive renin stimulation downregulates the h(P)RR.20

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Aliskiren-Binding Increases the Half Life of Renin and Prorenin in Rat Aortic Vascular Smooth Muscle Cells

Wendy W. Batenburg, René J.A. de Bruin, Jeanette M.G. van Gool, Dominik N. Müller, Michael Bader, Geneviève Nguyen and A. H. Jan Danser

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