Aliskiren Accumulates in Renin Secretory Granules and Binds Plasma Prorenin


Abstract—The vascular effects of aliskiren last longer than expected based on its half life, and this renin inhibitor has been reported to cause a greater renin rise than other renin-angiotensin system blockers. To investigate whether aliskiren accumulation in secretory granules contributes to these phenomena, renin-synthesizing mast cells were incubated with aliskiren, washed, and exposed to forskolin in medium without aliskiren (0.1 to 1000 nmol/L). (Pro)renin concentrations were measured by renin- and prorenin-specific immunoradiometric assays, and renin activity was measured by enzyme-kinetic assay. Without aliskiren, the culture medium predominantly contained prorenin, the cells exclusively stored renin, and forskolin doubled renin release. Aliskiren dose-dependently bound to (pro)renin in the medium and cell lysates and did not alter the effect of forskolin. The aliskiren concentrations required to bind prorenin were 1 to 2 orders of magnitude higher than those needed to bind renin. Blockade of cell lysate renin activity ranged from 27±15% to 79±5%, and these percentages were identical for the renin that was released by forskolin, indicating that they represented the same renin pool, ie, the renin storage granules. Comparison of renin and prorenin measurements in blood samples obtained from human volunteers treated with aliskiren, both before and after prorenin activation, revealed that ≤30% of prorenin was detected in renin-specific assays. In conclusion, aliskiren accumulates in renin granules, thus allowing long-lasting renin-angiotensin system blockade beyond the half-life of this drug. Aliskiren also binds to prorenin. This allows its detection as renin, and might explain, in part, the renin rise during renin inhibition. (Hypertension. 2008;52:1076-1083.)

Key Words: prorenin ■ renin ■ storage granule ■ mast cell ■ aliskiren

Renin inhibitors, like all renin-angiotensin system (RAS) blockers, increase the plasma concentration of renin because they attenuate the negative feedback effect of angiotensin (Ang) II on renin release.1,2 Whether they also increase kidney renin is still being debated.1-3 The rise in blood plasma has been suggested to be larger than during other types of RAS blockade,4 either because the degree of RAS blockade is superior during renin inhibition5 and/or because renin inhibitors increase the half-life of renin.6 It may also be an artifact, because renin inhibitors “activate” the precursor of renin, prorenin (Figure 1).7 This “activation” is because of the fact that the prorenin prosegment is capable of unfolding from the enzymatic cleft, in a pH- and temperature-dependent manner, thereby resulting in 2 prorenin conformations: a “closed,” inactive form and an “open” form that displays full enzymatic activity.8 Under physiological conditions, <2% of prorenin is in the open conformation.9 Renin inhibitors will bind to prorenin in the open conformation. Consequently, 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 the concentration of the inhibitor, a significant proportion of prorenin may be open (“nonproteolytic activation”), allowing its recognition by the active site-directed antibodies used in renin assays, despite the fact that the prosegment is still present (Figure 1).6,7,10

Unexpectedly, the vascular effects of aliskiren lasted much longer than expected on the basis of its circulating half-life.11,12 This could be because of accumulation of aliskiren-bound (pro)renin at tissue sites. In fact, long-lasting aliskiren accumulation has been demonstrated in the kidney,13 although the exact site of accumulation could not be established. An attractive hypothesis is that aliskiren reaches the renin storage sites in juxtaglomerular (JG) cells, thus allowing the release of blocked renin even when aliskiren is no longer present in blood. Unfortunately, JG cells loose their
capacity to store renin when cultured. However, this does not apply to the recently described renin-synthesizing human mast cells (HMC-1). These cells abundantly store renin and release prorenin constitutively and renin in a regulated manner, thereby closely mimicking the in vivo characteristics of JG cells.

In the present study, we set out, first, to investigate aliskiren accumulation in renin secretory granules in HMC-1 cells and, second, to determine to what degree the renin rise in humans postsiskiren actually represents prorenin. The latter was possible by applying to plasma samples of aliskiren-treated volunteers not only the widely applied renin immunoreactive assays but also a specific prorenin immunoreactive assay that is based on the recognition of the prosegment.

Methods

Cell Culture Studies

HMC-1 cells, developed from a patient suffering from mast cell leukemia, were a kind gift of Dr J.H. Butterfield (Mayo Clinic, Rochester, Minn). These cells abundantly store renin and release prorenin constitutively and renin in a regulated manner, thereby closely mimicking the in vivo characteristics of JG cells.

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Methods

Cell Culture Studies

HMC-1 cells, developed from a patient suffering from mast cell leukemia, were a kind gift of Dr J.H. Butterfield (Mayo Clinic, Rochester, Minn). Cells were seeded in 25-cm² culture flasks at a concentration of 10⁵ cells per milliliter and cultured for 7 days in 5 mL of supplemented Iscove’s modified Dulbecco’s medium containing 10% calf serum, in the absence or presence of aliskiren (0.1 nmol/L to 1.0 nmol/L, a kind gift of Novartis Pharmaceuticals, Basel, Switzerland) and/or 10 mmol/L of mannose 6-phosphate (M6P). Next, cells were separated from the culture medium by centrifugation at 1500 g for 5 minutes at room temperature. The culture medium was collected and stored at −20°C. The cells were washed in 10 mL of ice-cold Hanks’ balanced salt solution, centrifuged as described above, and, after removal of the supernatant, resuspended in fresh Iscove’s modified Dulbecco’s medium containing no aliskiren. Cells were then stimulated with the adenylyl cyclase activator forskolin (50 μmol/L; Sigma; dissolved in DMSO) or vehicle for 4 hours at 37°C. After the stimulation period, cells were separated from the stimulation medium by centrifugation at 1500 g, and the medium was collected and stored at −20°C. The pellet was resuspended in lysis buffer (0.2% Triton-X in PBS) and also stored at −20°C.

Human Studies

Plasma samples were obtained from 20 healthy subjects on a low-sodium diet (10 mmol daily sodium intake) each receiving 3 of 4 escalating doses of aliskiren (75, 150, 300, or 600 mg). Each subsequent aliskiren dose was given 2 days after the previous dose. Written informed consent was obtained from each patient, and the protocol was approved by the human subjects committee of the Brigham and Women’s Hospital and Harvard Medical School.

Biochemical Measurements

Renin and prorenin were measured in plasma, medium, and cell lysates by enzyme-kinetic assay and/or immunoradiometric assay (IRMA; Cisbio), using recombinant human (pro)renin as a control. To allow its measurement by renin IRMA, prorenin was activated in a nonproteolytic manner by incubating the sample for 48 hours with 10 μmol/L of aliskiren at 4°C. The renin inhibitor enters the enzymatic cleft in which the active site is located, thereby inducing a slow conformational change of the inactive (closed) form of the prorenin molecule into the active (open) form (Figure 1). This approach yields the same immunoreactive renin levels (“total renin”) as proteolytic prorenin activation with trypsin, yet without removing the prosegment. In a select set of samples, prorenin was also measured on the basis of its prosegment, replacing the ³¹I-labeled, active site–directed monoclonal antibody of the Cisbio kit by a prosegment-directed, ³¹I-labeled monoclonal antibody (F258-37-B1) in the IRMA (“prorenin IRMA”). F258-37-B1 is...
directed against the C-terminal part (p20 to p43) of the propeptide and does not react (<0.1%) with renin. F258-37-B1 also does not react (<0.1%) with intact, inactive prorenin. 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. Detection limits of the enzyme-kinetic assay, the renin IRMA, and the prorenin IRMA were 0.1 ng of Ang I per milliliter per hour, 1 pg of renin per milliliter, and 5 pg of prorenin per milliliter, respectively. Angiotensinogen in cell lysates and medium was measured as described previously (detection limit: 0.1 pmol/mL). Protein was measured according to the Bradford method.

**Data Analysis**

Results are expressed as means±SEMs. IC50 values were calculated as described previously. Ang I–generating activities obtained in the enzyme-kinetic assay were converted to renin concentrations based on the fact that 1 ng of Ang I per milliliter per hour corresponds with 2.6 pg of human renin per milliliter. Statistical analysis was performed using 1-way ANOVA. P<0.05 was considered significant.

**Cell Culture Studies**

**Immunoreactive (Pro)renin Levels**

In the absence of aliskiren, HMC-1 cells contained immunoreactive renin (Figure 2A), as measured with the Cisbio renin IRMA. The cellular total renin levels (ie, the immunoreactive renin levels measured by Cisbio renin IRMA after prorenin activation) were identical to the immunoreactive renin levels without prorenin activation, suggesting that the cells did not store prorenin. Indeed, when using a prosegment-directed antibody, no signal was detected in the cell lysates (Figure 2B).

The medium of nonaliskiren-exposed cells also contained immunoreactive renin. These levels increased ≈5-fold after prorenin activation (Figure 2C). This suggests that the cells predominantly released prorenin. The prosegment assay data confirmed this view (Figure 2D). Neither the medium nor the cells contained detectable amounts of angiotensinogen (n=4), thus demonstrating that HMC-1 cells cannot generate Ang II independently.

A 7-day incubation with aliskiren increased the cellular renin levels in a concentration-dependent manner (Figure 2A). The maximum increase in renin, reached at 1 μmol/L of aliskiren, was ≈4-fold. Prorenin could not be detected in the cells at any of the tested aliskiren concentrations (Figure 2B). To investigate the possibility that the aliskiren-induced rise in cellular renin was because of the reuptake of medium renin via M6P receptors (as part of a clearance process), the experiments were repeated in the presence of excess M6P. The maximum aliskiren-induced increase in cellular renin (2.8±0.2-fold; n=3) was unaltered in the presence of M6P (2.1±0.4-fold; n=3), and M6P did not alter the cellular renin levels in the absence or presence of aliskiren (122±22% and 88±14% of the levels without M6P, respectively; P value not significant versus 100%). Therefore, the aliskiren-induced increase in cellular renin is not because of reuptake of medium renin via M6P receptors.

**Results**

**Figure 2.** Levels of immunoreactive renin, total renin (=immunoreactive renin after prorenin activation), and prosegment-containing prorenin in the cell lysates (A and B) and culture medium (C and D) of HMC-1 cells after a 7-day incubation in the absence (C) or presence of aliskiren (0.1 nmol/L to 1.0 μmol/L). Immunoreactive renin was measured with an antibody directed against the active site, whereas prosegment-containing prorenin was measured with an antibody against the prosegment. Data are means±SEMs of n=8. #P<0.05 vs total renin control; *P<0.05 vs corresponding immunoreactive renin.

In the medium, aliskiren also increased renin in a concentration-dependent manner (Figure 2C). This increase was much larger (maximally ≈20-fold) than that in the cells (P<0.01) and unaffected by M6P (data not shown). In parallel with this rise in secreted renin, the effect of prorenin activation on medium renin immunoreactivity diminished until, at aliskiren concentrations of 0.1 μmol/L, prorenin activation no longer yielded an increase in medium renin immunoreactivity. Yet, the prosegment assay still allowed the detection of intact, prosegment-containing prorenin (Figure 2D). This indicates that the 7-day incubation of the cells with aliskiren had resulted in nonproteolytic activation of prorenin, allowing its detection in both the Cisbio renin IRMA and the prosegment assay. Apparently, aliskiren concentrations of 0.1 μmol/L were sufficient to fully open all of the prorenin molecules in the medium during a 7-day incubation. Because prorenin activation in nonaliskiren-exposed cells resulted in an ≈5-fold rise of renin immunoreactivity, the 20-fold rise in renin should be corrected for this detection of prorenin as renin. This implies that the true aliskiren-induced rise in medium renin is ≈4-fold, ie, identical to the aliskiren-induced rise in cellular renin. Forskolin doubled renin release both in the absence and presence of aliskiren (P<0.01; Figure 3).
4 hours after a 7-day incubation of the cells in the absence (C) or presence of aliskiren (0.1 nmol/L to 1.0 μmol/L). Data are means±SEMs of n=5. Forskolin doubled the renin release at all of the aliskiren concentrations (P<0.01).

Figure 3. Immunoreactive renin levels in the stimulation medium of HMC-1 cells exposed to 50 μmol/L of forskolin for 4 hours after a 7-day incubation of the cells in the absence (C) or presence of aliskiren (0.1 nmol/L to 1.0 μmol/L). Data are means±SEMs of n=5. Forskolin doubled the renin release at all of the aliskiren concentrations (P<0.01).

Renin Activity
Both cell lysate and culture medium contained Ang I–generating activity in the absence of aliskiren, and the activity in the culture medium was ≈10-fold lower than that in the cells (Figure 4, left and middle panels). Aliskiren, when incubated for 7 days with the cells, did not alter the Ang I–generating activity in the cell lysates (Figure 4, left). It did, however, concentration-dependently reduce the Ang I–generating activity in the culture medium (Figure 4, middle; P<0.01). To verify whether aliskiren had been degraded by the cells, Ang I–generating activity was also measured in culture medium of control cells (ie, cells that had not been incubated with aliskiren) after adding aliskiren to the incubation sample. As shown in Figure 4 (middle), the aliskiren-induced inhibition of Ang I–generating activity was identical under both conditions (IC50 3.5±1.2 versus 1.2±0.4 nmol/L; P value not significant). Thus, a 7-day incubation had not degraded aliskiren.

Stimulating the cells with forskolin resulted in the appearance of Ang I–generating activity in the stimulation medium (Figure 4, right). Applying forskolin after the 7-day incubation of the cells with aliskiren revealed a concentration-dependent decrease in the released amount of Ang I–generating activity (P<0.05), despite the fact that the forskolin studies were performed without adding aliskiren again to the medium. Moreover, when incubating the stimulation medium with excess human renin, the Ang I–generating activity of this renin was 110±10% of that without stimulation medium (n=5). This indicates that the stimulation medium no longer contained aliskiren concentrations capable of blocking HMC-1 cell-released renin, ie, that the wash procedure had effectively removed aliskiren.

The renin activity data should be viewed in light of the increased immunoreactive renin levels during aliskiren exposure. Figure 5, therefore, displays the amount of aliskiren-bound renin (ie, inhibited renin) as a percentage of the total amount of renin in the cell lysate, culture medium, and stimulation medium (ie, 100%×[1−(Ang I–generating activity×2.6)/immunoreactive renin]). It then becomes clear that aliskiren has also blocked intracellular renin. In fact, the curves for cell lysate renin (Figure 5, left) and renin in the stimulation medium (Figure 5, right) were identical (P value not significant). This demonstrates that they represent the same renin pool, ie, the renin storage granules.

In the culture medium (Figure 5, middle), a distinction was made between renin and prorenin. The prosegment assay data had indicated that the majority of culture medium total renin represented prorenin, both without and with aliskiren (Figure 2D). It was, therefore, assumed that, at all aliskiren concentrations, the percentage of total renin that represented renin was identical to that in the absence of aliskiren. The Ang I–generating activity data were compared with the renin levels that were, thus, calculated, to determine the amount of aliskiren-bound renin in the culture medium. The remainder of the total renin levels represented prorenin. To also calculate the amount of aliskiren-bound prorenin, these levels were compared with the amount of prorenin that was directly recognized in the renin IRMA, because this recognition is

Figure 4. Levels of renin activity in the cell lysates, culture medium, and stimulation medium of HMC-1 cells after a 7-day incubation of the cells in the absence (C) or presence of aliskiren (0.1 nmol/L to 1.0 μmol/L) and a subsequent stimulation with 50 μmol/L of forskolin for 4 hours. The middle panel also shows the renin activity of control medium to which aliskiren was added during the assay ("aliskiren added," vs "aliskiren present," which represents the medium obtained after the 7-day incubation with aliskiren). Data are means±SEMs of n=5 to 8.
based on the aliskiren-induced nonproteolytic activation of prorenin, ie, on the binding of aliskiren to prorenin. As can be seen in Figure 5 (middle), the aliskiren concentrations required to bind prorenin were 1 to 2 orders of magnitude higher than the concentrations needed to bind renin.

Human Plasma Samples

Figure 6 shows the immunoreactive (pro)renin levels in plasma of human subjects treated with aliskiren. These values are based on the Cisbio renin IRMA measurements before and after prorenin activation. According to these measurements, aliskiren treatment increased plasma renin in a dose-dependent manner, and no increases in prorenin occurred. If anything, with this assay, prorenin even decreased at t=5 hours after the intake of the 2 highest aliskiren doses. In contrast, when measuring prorenin based on the presence of its prosegment (“true” prorenin), prorenin increases were observed, which were most prominent at 24 hours after the intake of aliskiren. The difference between the 2 prorenin measurements became significant at aliskiren doses of ≥150 mg, and prorenin even appeared to decrease at 5 hours after the intake of aliskiren. The direct prorenin measurements now show that this is incorrect and that, in fact, prorenin increases after the intake of aliskiren. This increase was of modest proportion in comparison with the increase in renin. Furthermore, it was most apparent at 24 hours after intake, as opposed to the rise in renin that was already maximal at 5 hours. These data are in full agreement with the fact that prorenin and renin release occur in a constitutive and regulated manner, respectively, and that RAS stimulation predominantly causes a rise in renin.23

The mechanism underlying the underestimation of prorenin when using renin IRMAs involves aliskiren binding to prorenin in the open conformation, thereby preventing its return to the closed conformation.6 Only the former conformation will be recognized in renin IRMAs, and, thus, renin will be overestimated and prorenin underestimated when using these assays. Under normal physiological conditions, <2% of prorenin is in the open conformation, because at 37°C the inactivation step occurs rapidly.7 Because at low temperature the equilibrium shifts considerably toward open prorenin, one may argue that the observations in this study are, at most, ex vivo phenomena related to frozen storage of the samples. However, the aliskiren effect was most apparent at 24 and 48 hours after drug intake, and not at 5 hours after intake, when the plasma aliskiren levels are highest (and when the largest consequences of this phenomenon, if solely occurring during storage, should have occurred). This, therefore, suggests that the aliskiren binding to prorenin had already occurred in vivo.

Discussion

This study reveals, first, that aliskiren accumulates in renin storage sites in renin-synthesizing cells and, second, that a significant percentage of the in vivo renin rise after aliskiren is in fact nonproteolytically activated, intact prorenin. The latter has been suggested before,10 and our data are the first to demonstrate the dose dependency of this phenomenon and to suggest that it also occurs in vivo. At low aliskiren doses, direct prorenin measurements (based on the detection of the prosegment) yielded results that were identical to the indirect prorenin measurements (calculated from the difference between the results of the renin IRMA before and after prorenin activation). However, at aliskiren doses of ≥150 mg, the indirect measurements yielded lower prorenin levels, and prorenin even appeared to decrease at 5 hours after the intake of aliskiren. The direct prorenin measurements now show that this is incorrect and that, in fact, prorenin increases after the intake of aliskiren. This increase was of modest proportion in comparison with the increase in renin. Furthermore, it was most apparent at 24 hours after intake, as opposed to the rise in renin that was already maximal at 5 hours. These data are in full agreement with the fact that prorenin and renin release occur in a constitutive and regulated manner, respectively, and that RAS stimulation predominantly causes a rise in renin.23
In further support of this concept, aliskiren, when incubated with HMC-1 cells at 37°C for 7 days, concentration-dependently blocked both renin and prorenin in the medium, until, at concentrations of ≥0.1 μmol/L, virtually all of the renin and prorenin had become aliskiren bound. Such aliskiren concentrations do occur in vivo (Figure 6), and, thus, these results are clinically relevant. The concentrations required to fully block prorenin were 1 to 2 orders of magnitude higher than the concentrations needed to block renin. This relates to the small percentage of prorenin that is in the open conformation at 37°C. Because this percentage is higher at lower temperatures, it is much easier to “activate” prorenin with aliskiren at 4°C than at 37°C (ie, to fully shift the equilibrium between the closed and open conformation into the direction of the open, renin IRMA-recognizable conformation). Aliskiren binding to both renin and prorenin stabilizes the molecule, thereby increasing the half-life by a factor of 2 to 3. This explains why the medium and cellular levels of (pro)renin in the present study increased progressively when incubating the cells with increasing concentrations of aliskiren. In vivo, interference with the negative feedback loop would have been proposed to explain a rise in (pro)renin. However, because we were unable to demonstrate angiotensinogen synthesis by these cells, this explanation cannot be applied here. In fact, incubation with either captopril (10 μmol/L) or the Ang II type 1 receptor antagonist eprosartan (1 μmol/L) for 7 days did not increase renin (n = 3; data not shown).

Finally, when exposing HMC-1 cells to forskolin after their 7-day incubation with aliskiren, the cells released aliskiren-bound renin. Thus, apparently, aliskiren is taken up by HMC-1 cells and reaches renin granules. Indeed, it has been suggested in both mast cells and JG cells that early secretory granules have the ability to take up extracellular renin.
substances, delivered through retrograde transport via early/recycling endosomes and surcompassing the Golgi network. This is perhaps not surprising considering that the renin granules originate as lysosomes. Experiments with excess M6P excluded the possibility that the cellular uptake of aliskiren represented internalization of aliskiren-bound renin from the medium via M6P receptors, which are known clearance receptors for both renin and prorenin. In vivo studies in rats have already indicated that, 3 weeks after stopping aliskiren treatment, when plasma levels are below the detection limit, the renal tissue levels of aliskiren are still well above its IC50. When combining these data with the present results, it seems reasonable to suggest that the renal “reservoir” of aliskiren is in fact the JG cell. Thus, these observations might explain why the effects of aliskiren on blood pressure and renin activity last for several weeks after stopping treatment.

Perspectives

The cell culture and clinical data in the present study, when combined, convey 2 important messages. First, aliskiren binds intracellularly to stored renin, thus inhibiting the enzymatic activity of this enzyme before its secretion. The actual presence of aliskiren-bound renin in storage granules implies that the effect of the drug will continue even when drug levels in blood are low or undetectable (eg, when a patient does not take his daily dose or when treatment has been stopped altogether). This phenomenon might, therefore, contribute to the long-lasting effects of aliskiren after stopping treatment. Obviously, because our results were obtained in mast cells, future in vivo studies should now confirm aliskiren accumulation in JG cells, eg, using fluorescently tagged aliskiren.

Second, aliskiren also binds to prorenin. The cell culture data show that the concentrations required to accomplish this, at least at 37°C, are higher than those required for its binding to renin. Nevertheless, such concentrations do occur in blood during treatment, and, thus, this phenomenon is of clinical relevance. On the one hand, aliskiren binding allows prorenin to be detected as renin in the commercially available renin IRMAs, and it, thus, explains, at least in part, the large rise in renin that has been noted during aliskiren treatment. Simultaneously, given the tight binding of the molecule, it would allow aliskiren-bound prorenin to accumulate at tissue sites. Because prorenin, via binding to the (pro)renin receptor, has been suggested to be a major contributor to tissue Ang production, this would imply that aliskiren highly efficiently blocks tissue Ang generation. Such efficient interference with tissue Ang generation might, eg, explain why the effects of aliskiren on renal plasma flow are much bigger than those of other RAS blockers. In view of the long half-life of aliskiren-bound prorenin, it might also underlie the long-lasting effects of aliskiren after stopping treatment.

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Disclosures

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