Handle Region Peptide Counteracts the Beneficial Effects of the Renin Inhibitor Aliskiren in Spontaneously Hypertensive Rats

Joep H.M. van Esch, Richard van Veghel, Ingrid M. Garrelts, Frank Leijten, Angelique M. Bouhuizen, A.H. Jan Danser

Abstract—To investigate whether the putative (pro)renin receptor blocker, the handle region peptide (HRP), exerts effects on top of the blood pressure–lowering and cardioprotective effects of the renin inhibitor aliskiren, spontaneously hypertensive rats were implanted with telemetry transmitters to monitor heart rate and mean arterial pressure (MAP). After a 2-week recovery period, vehicle, aliskiren, HRP (100 mg/kg per day), and HRP + aliskiren were infused for 3 weeks using osmotic minipumps. Subsequently, the heart was removed to study coronary function according to Langendorff. Baseline MAP and heart rate in vehicle-treated rats were 146±3 mm Hg and 326±4 bpm. HRP did not affect MAP, whereas aliskiren and HRP + aliskiren lowered MAP (by maximally 29±2 and 20±1 mm Hg, respectively) without affecting heart rate. Aliskiren significantly reduced MAP throughout the 3-week infusion period, whereas the blood pressure–lowering effect of HRP + aliskiren returned to baseline within 2 weeks of treatment. In comparison with vehicle, aliskiren increased the endothelium-dependent response to bradykinin and decreased the sensitivity to angiotensin (Ang) II (most likely because of a wide variety of doses that has been applied, ranging from 0.1 mg/kg per 28 days to 1.0 mg/kg per day). In SHRs, focusing on blood pressure, cardiac function, and cardiac hypertrophy in an angiotensin-independent manner. (Hypertension. 2011;57:00-00.)

Key Words: handle region peptide ■ (pro)renin ■ aliskiren ■ spontaneously hypertensive rat ■ angiotensin

The renin inhibitor aliskiren exerts beneficial effects in the heart of spontaneously hypertensive rats (SHRs) and in the mouse heart postmyocardial infarction. Among others, it improved coronary endothelial function, diminished the responsiveness to angiotensin (Ang) II (most likely because of a downregulation of Ang II type 1 [AT1] receptor density), reduced cardiomyocyte area, and prevented remodeling. These observations parallel findings in humans, where aliskiren reduced left ventricular mass reduction at least as effectively as other blockers of the renin–angiotensin system (RAS). However, although the suppression of cardiac Ang II by aliskiren was superior to that induced by Ang-converting enzyme inhibition, Ang II levels did not decrease to 0. In part, this could relate to the fact that the human renin inhibitor aliskiren only modestly blocks rat renin. In addition, tissue Ang generation may involve prorenin, which displays activity when bound to its receptor, the (pro)renin receptor ([P]RR). Although aliskiren theoretically will block such prorenin-dependent Ang I generation, an alternative way to suppress this Ang source is the infusion of the (P)RR blocker called the handle region peptide (HRP). Its effectiveness in vivo is controversial, in part because of a wide variety of doses that has been applied, ranging from 0.1 mg/kg per 28 days to 1.0 mg/kg per day. HRP will also block the direct, Ang-independent effects of prorenin when bound to the (P)RR and may, thus, suppress cardiac fibrosis. In the present study, we set out to investigate the effect of HRP (1 mg/kg per day), alone or on top of aliskiren, in SHRs, focusing on blood pressure, cardiac function, and tissue Ang content.

In Vivo Studies

Male SHRs (280 to 300 g; n=40), obtained from Charles River, were housed in individual cages and maintained on a 12-hour light/dark cycle, having access to standard laboratory rat chow and water ad libitum. All of the experiments were performed under the regulation and permission of the Animal Care Committee of the Erasmus MC. Radiotelemetry transmitters were implanted as described before. Nine days later, baseline telemetry measurements (mean arterial pressure [MAP] and heart rate [HR]) were obtained during 2 days. Then (day 0) osmotic minipumps (2ML4 ALZET) were implanted...
subcutaneously under isoflurane anesthesia to infuse vehicle (saline), aliskiren (a gift of Novartis, 100 mg/kg per day), and/or rat HRP (NH2- RILLKKMPSV-COOH, Biosynth, 1 mg/kg per day). In the animals receiving 2 drugs, 2 separate minipumps were implanted at both sides of the body. After 3 weeks of infusion, animals were anesthetized by inhalation of isoflurane, and the hepatic portal vein was cannulated to collect blood for the measurement of plasma renin activity (PRA) and Angs. Hearts and kidneys were excised and placed in ice-cold Tyrode buffer or stored at −80°C.

In Vitro Studies and Histology
Hearts were buffer perfused according to Langendorff, as described previously. Coronary flow (CF) was measured with a flow probe (Transonic Systems). After a stabilization period of 30 minutes, baseline values of CF were obtained. Next, bolus injections (100 μL) of Tyrode buffer were applied 3 times to determine injection-induced changes in CF. Concentration-response curves to bradykinin and Ang II were constructed by bolus injections, in the absence or presence of the Ang II type 2 receptor antagonist PD123319 (1 μmol/L), after which the maximum CF was determined by injecting 10 mmol/L of sodium nitroprusside. Next, the hearts were collected, and the ventricular heart weight was determined after removal of the atria and large vessels to allow the calculation of the heart weight/body weight ratio. Ventricles were then cut into 3 transversal sections and fixed in a 3.5% to 4.0% formaldehyde solution. After fixation, sections were dehydrated and paraffin embedded. Gomori silver staining was applied to deparaffinized 5-μm-thick sections of the left ventricle to visualize individual cardiomyocytes.

Biological Measurements
PRA and Angs were measured as described before. Renal AT1 receptor protein content was quantified by Western blot analysis. In brief, kidneys were homogenized with a Polytron PT2100 (Kinematica AG) in ice-cold Lysis buffer. SDS-PAGE was performed on 10% polyacrylamide gels. Each lane was loaded with 20 μg of protein. After transfer, nonspecific sites were blocked with 5% milk in PBS-Tween. Then, the blots were incubated overnight with 1:400 anti-AT1 receptor (N-10; Santa Cruz Biotechnology) and 1:20.000 anticlin (C4; Millipore). After washing, the sites of the antibody-antigen reaction were visualized with 1:5000 horseradish peroxidase–conjugated secondary antibodies (Bio-Rad Laboratories) using the enhanced chemiluminescence Western blotting detection system (Pierce Biotech). Signal intensities were quantified by scanning densitometry (Bio-Rad Laboratories).

Quantitative Real-Time RT-PCR
Total RNA was isolated from kidneys using the TRIzol reagent (Gibco-BRL) and reverse transcribed. The resulting cDNA was amplified in 40 cycles (denaturation at 95°C for 10 minutes, thermal cycling at 95°C for 15 seconds, and annealing/extension at 60°C for 1 minute) with a Step-One cycler (NYSE, Applied Biosystems) using the SYBR Green quantitative PCR core kit (Eurogentec). Primers (forward 5'-ACTGCTGGAACCACTCTGTTC-3', reverse 5'-TCG/TGACAGCGCTTGATGCG-3') were from Invitrogen. The comparative cycle time method (ΔΔCT) was used for relative quantification of gene expression. Messenger RNA expression was normalized versus actin and expressed as the ratio of target to control value.

Data Analysis
Telemetric data were recorded and digitalized using the Datequest Acquisition and Analysis system (DQ ART 3.1 Silver, Datascience Inc). Each animal was sampled for 10 seconds at 10-minute intervals for a period of 23 days. All of the recordings were averaged per day, and baseline values were calculated using the data from the first 2 days of measurement before treatment was started. Changes in blood pressure from baseline were analyzed by comparison of the areas over the curve, as calculated by the trapezoidal method (millimeters of mercury×days). Data obtained with the Langendorff preparation were recorded and digitalized using WinDaq waveform recording software (Dataq Instruments). After manual selection of the desired signals preinjection and postinjection, data were analyzed using Matlab (Mathworks Inc). Six consecutive beats were selected for determination of CF.

Statistical analysis between groups was performed by Student t test or 1-way ANOVA, followed by post hoc evaluation according to Dunnet. P<0.05 was considered significant. The 4 groups described in this study were part of a larger study including 2 additional groups of rats treated with an Ang-converting enzyme inhibitor or an AT1 receptor antagonist. These data have been published before.

Results

Hemodynamics
Neither vehicle nor HRP affected MAP or HR (Table and Figure 1). Aliskiren lowered MAP, without affecting HR. MAP was maximally reduced at the fourth day after the start of infusion. Thereafter, the effect of aliskiren leveled off, but MAP remained reduced (P<0.05) at days 7, 14, and 21. HRP, when given in combination with aliskiren, greatly diminished its effect on MAP, without altering HR, and after 14 days, MAP in the HRP+aliskiren-treated animals was identical to that in vehicle-treated rats.

PRA and Angs
HRP did not significantly alter PRA, plasma Angs, or tissue Angs, either alone or on top of aliskiren (Table).

Langendorff Studies
Baseline CFs of vehicle-treated SHRs (12±0.8 mL/min; n=9), aliskiren-treated SHRs (9.6±0.6 mL/min; n=10), HRP-treated SHRs (9.5±1.1 mL/min; n=6), and HRP+aliskiren-treated SHRs (13±1.5 mL/min; n=5) were identical. Bolus injections with Tyrode buffer injections did not significantly affect CF (Figure 2). In comparison with vehicle-treated SHR, aliskiren treatment increased the effect of bradykinin (P<0.05; Figure 2A) and decreased the effect of Ang II (P<0.05; Figure 2D). These altered responses to bradykinin and Ang II were abolished when HRP was added on top of aliskiren (Figure 2C and 2F, respectively). HRP alone did not alter the response to bradykinin and Ang II in comparison with vehicle-treated SHRs (Figure 2B and 2E, respectively).

PD123319 did not significantly increase the response to Ang II in the aliskiren-treated rats (n=5; P=0.09, data not shown). The maximum CF response was 26±2.3 mL/min, and treatment with aliskiren, HRP, or HRP+aliskiren did not alter this response (data not shown).

Renal AT1 Receptor Expression
Aliskiren treatment reduced the renal AT1 receptor content (P<0.05; Figure 3, middle), and a similar tendency was observed for AT1 receptor gene expression (P value not significant; Figure 3, bottom). Treatment with HRP, both with and without aliskiren, increased AT1 receptor gene expression (P<0.05; Figure 3, bottom), but only in the presence of aliskiren did this result in a marginal (P value not
significant) increase in renal AT$_1$ receptor content (Figure 3, middle) versus aliskiren alone.

**Cardiac Hypertrophy**

Treatment of SHR with aliskiren, HRP, or HRP+aliskiren did not significantly alter the heart weight/body weight ratio (Table). Aliskiren reduced ($P<0.05$) the cardiomyocyte area in the left-ventricular wall (Figure 4). HRP, when given on top of aliskiren, reversed this effect and did not affect cardiomyocyte area when given alone.

**Discussion**

The present study is the first to show that HRP counteracts the beneficial cardiac effects of the renin inhibitor aliskiren. The most likely explanation for this finding is that HRP annihilated the blood pressure–lowering effect of aliskiren, thereby preventing the improvement of coronary endothelial function and cardiomyocyte hypertrophy that depend, at least in part, on the decrease in blood pressure.

In agreement with a previous study in stroke-prone SHRs,$^1$ HRP, when infused alone, did not affect blood pressure or

---

**Table. HR and MAP at Baseline; the Maximum Decrease in MAP; Maximum Decrease in MAP at Days 7, 14, and 21 After the Start of Treatment; the Area Over the Curve; and Body Weight, Heart Weight, Heart Weight/Body Weight Ratio, PRA, Ang I, and Ang II Levels in Blood Plasma and Kidney and in SHRs Treated for 3 Weeks With Vehicle, Aliskiren, HRP, or HRP+Aliskiren**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle, Control</th>
<th>Aliskiren, 100 mg/kg per Day</th>
<th>HRP, 1 mg/kg per Day</th>
<th>HRP+Aliskiren, 1 mg/kg per Day+100 mg/kg per Day</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>10</td>
<td>12</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Baseline HR, bpm</td>
<td>326±4</td>
<td>328±4</td>
<td>315±2</td>
<td>317±4</td>
</tr>
<tr>
<td>Baseline MAP, mm Hg</td>
<td>146±3</td>
<td>151±3</td>
<td>144±1</td>
<td>139±2</td>
</tr>
<tr>
<td>$\Delta$MAP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum</td>
<td>$-1±1$</td>
<td>$-29±2^*$</td>
<td>8±1</td>
<td>$-20±1^\dagger$</td>
</tr>
<tr>
<td>Day 7</td>
<td>$-3±1$</td>
<td>$-22±3^*$</td>
<td>3±1</td>
<td>$-13±1^\dagger$</td>
</tr>
<tr>
<td>Day 14</td>
<td>$3±1$</td>
<td>$-14±2^*$</td>
<td>2±1</td>
<td>$-4^\dagger$</td>
</tr>
<tr>
<td>Day 21</td>
<td>$4±1$</td>
<td>$-10±3^*$</td>
<td>7±1</td>
<td>$-1±2$</td>
</tr>
<tr>
<td>AOC MAP, mm Hg×d</td>
<td>68±10</td>
<td>388±36*</td>
<td>83±12</td>
<td>192±15$^\dagger$</td>
</tr>
<tr>
<td>BW, g</td>
<td>385±4</td>
<td>360±4$^*$</td>
<td>377±10</td>
<td>373±8</td>
</tr>
<tr>
<td>HW, g</td>
<td>1.39±0.04</td>
<td>1.29±0.04</td>
<td>1.37±0.05</td>
<td>1.34±0.02</td>
</tr>
<tr>
<td>HW/BW, g/kg</td>
<td>3.61±0.08</td>
<td>3.57±0.09</td>
<td>3.61±0.06</td>
<td>3.61±0.06</td>
</tr>
<tr>
<td>PRA, pmol of Ang I per mL per h</td>
<td>10.1 (2.8 to 29.4)</td>
<td>3.7 (0.4 to 28.0)</td>
<td>7.8 (3.4 to 17.2)</td>
<td>7.1 (4.7 to 10.8)</td>
</tr>
<tr>
<td>Plasma Ang I, fmol/mL</td>
<td>165 (32.5 to 989)</td>
<td>71.1 (10.0 to 515)</td>
<td>64.2 (27.6 to 120)</td>
<td>66.7 (32.5 to 239)</td>
</tr>
<tr>
<td>Plasma Ang II, fmol/mL</td>
<td>44.2 (8.6 to 91.1)</td>
<td>30.5 (4.2 to 111)</td>
<td>13.1 (3.3 to 22.6)</td>
<td>15.4 (8.4 to 25.2)</td>
</tr>
<tr>
<td>Kidney Ang I, fmol/g</td>
<td>772 (201 to 3350)</td>
<td>424 (113 to 2144)</td>
<td>229 (135 to 419)</td>
<td>259 (212 to 346)</td>
</tr>
<tr>
<td>Kidney Ang II, fmol/g</td>
<td>200 (54.4 to 942)</td>
<td>242 (77.8 to 926)</td>
<td>210 (111 to 372)</td>
<td>197 (172 to 284)</td>
</tr>
</tbody>
</table>

Data are shown as mean±SEM unless otherwise specified. PRA, Ang I, and Ang II levels are shown as geometric mean (range). $\Delta$MAP indicates maximum decrease in MAP; AOC, area over the curve; BW, body weight; HW, heart weight.

$^*P<0.05$ vs SHR vehicle control.

$^\dagger P<0.05$ vs SHR aliskiren 100 mg/kg per day.
cardiac Ang II levels. Aliskiren suppressed the renal Ang II formation, even when the cardiac Ang levels remained suppressed during aliskiren treatment, even after stopping treatment.4 However, the aliskiren concentrations required to block rat renin are approximately 100 times higher than those needed to block human renin,23 and, thus, such accumulation, even if occurring in the renin storage granules,24 will only be of significance in humans and animals whose renin is blocked with high potency by aliskiren (eg, the marmoset and the mouse, but not the rat).

HRP represents 10 amino acids of the prosegment and its binding to the (P)RR.25 However, in a previous study we were unable to detect prorenin activating/blocking effects of HRP, nor did this drug act as a renin inhibitor, at least at concentrations ≤1 μmol/L.5 Such high concentrations are unlikely to be reached at the infusion rate applied in this study.10,26 Nevertheless, PRA in the present study tended to be lower in the HRP-treated animals, although this did not reach significance. Possibly therefore, given the well-known inverse relationship between renin and blood pressure, the lower PRA simply reflects the rise in MAP.

Unexpectedly, HRP increased renal AT₁ receptor gene expression. This tended to counteract (P value not significant) the aliskiren-induced decrease in tissue AT₁ receptor content. Such AT₁ receptor downregulation has been observed before in several studies27,28 and may help to explain the beneficial effect of aliskiren in the absence of clear Ang II reductions. Clearly, however, this decrease is not the only determinant of the reduced coronary Ang II

**Figure 2.** Effect of bradykinin (BK; A through C) and Ang II (D through F) bolus injections (100 μL) on CF after a 3-week treatment of SHR with vehicle, aliskiren, HRP, or HRP + aliskiren. Data (mean ± SEM of 4 to 11 experiments) were obtained using the Langendorff heart preparation and represent the percentage of change from baseline. The concentrations on the x axis represent the concentration in the injection fluid. T represents the effect of a bolus injection with Tyrode buffer. Data after treatment with vehicle and aliskiren are redrawn from van Esch et al.1 *P < 0.05 vs vehicle-treated SHR.
responsiveness after aliskiren treatment. An alternative explanation involves the upregulation of vasodilatory Ang II type 2 receptors. If present, PD123319 should have increased the coronary Ang II response in aliskiren-treated SHRs, as it does in Wistar-Kyoto rats (but not untreated SHRs).12,29 Yet, the PD123319-induced potentiation after aliskiren treatment was not significant.

Because HRP and aliskiren were infused with separate minipumps, there is no reason to assume that a kinetic interaction between the 2 drugs has somehow inactivated aliskiren. The lack of a significant effect of HRP on the aliskiren-induced changes in PRA also argues against this concept. In summary, our data do not support an Ang-involving interaction by HRP that could explain its countering effects. At most, HRP tended to diminish the aliskiren-induced decrease in AT1 receptor content, but this was not significant and was unrelated to changes in Ang levels, because HRP even increased AT1 receptor expression in the absence of any change in renin or Ang.

Possibly, therefore, HRP may have blocked Ang-independent effects occurring after prorenin-(P)RR binding. Such effects are more likely to occur after RAS blockade, because this results in a rise of both renin and prorenin. Whether HRP also blocks renin-induced effects via the (P)RR remains a matter of debate. It is important to realize that the aliskiren-induced rises in renin (≥10- to 15-fold) and prorenin (≈2- to 3-fold)24,30 are far below those occurring in prorenin-expressing transgenic animals (up to several orders of magnitude).31–33 The phenotype of such animals, if any, appeared to depend on Ang generation, because the modest changes were fully normalized by captopril treatment.31 HRP was ineffective in these animals.31 Simultaneously, (P)RR-overexpressing rats did not display changes in RAS components, although they did develop hypertension, proteinuria, and glomerulosclerosis with aging.34,35 The latter 2 could be blocked by HRP treatment, suggesting that (P)RR activation, per se, that is, independent of the RAS, has deleterious effects. In apparent contrast with the view that (P)RR blockade is beneficial, a recent study revealed that cardiomyocyte-specific ablation of the (P)RR results in lethal heart failure, mainly because it created a loss-of-function model for vacuolar H⁺-ATPase.36 Whether HRP affects the (P)RR for vacuolar H⁺-ATPase connection is currently unknown. Our data support the concept that (P)RR blockade is detrimental, at least on top of renin inhibition. Opposing effects of HRP have been reported before, for example, decreases in renal...
extracellular signal–regulated kinase 1/2 phosphorylation (resulting in diminished glomerulosclerosis in diabetic mice)\textsuperscript{22} and increases in ocular extracellular signal–regulated kinase 1/2 phosphorylation (causing neuronal and glia injury in the retina),\textsuperscript{26} and, thus, a unifying concept might be that HRP acts as a partial agonist.

Finally, given the low HRP levels in vivo (<1 nmol/L), even when infused at a rate of 1 mg/kg per day, as opposed to the >1 μmol/L levels that are required in vitro to block prorenin-induced effects,\textsuperscript{37} the effects of HRP may go beyond those of (P)RR activation/blockade. Wilkinson-Berka et al\textsuperscript{26} have suggested that HRP modulates cytokine release or cells of the immune system at the site of infusion. This needs to be investigated further. Preliminary data in isolated rat iliac arteries do not support direct constrictor effects of HRP (n = 3, J.H.M. van Esch, unpublished results, 2010).

Perspectives

Studies in transgenic rodents overexpressing prorenin or the (P)RR question the importance of the prorenin-(P)RR interaction. The present study does not reveal Ang-suppressing effects of HRP, even when infused at high doses. The hypertensive effects of HRP on top of aliskiren, combined with the lethal cardiac phenotype of cardiomyocyte-specific ablation of the (P)RR observed by others, raises concern regarding the use of HRP in cardiovascular patients, despite its beneficial effects in diabetic nephropathy and retinopathy. Clearly, a detailed insight into its mechanism of action is needed to establish to what degree HRP exerts effects that are unrelated to the (P)RR. Importantly in this regard, Leckie and Bottrell were unable to demonstrate a specific binding site for HRP on human endothelial cells,\textsuperscript{38} and HRP binding also occurred in cells not expressing the (P)RR.\textsuperscript{39}

Sources of Funding

This study was supported by a grant of the Netherlands Heart Foundation (NHS2007B019).

Disclosures

A.H.J.D. has received research support from and has consulted for Novartis.

References

2. Westermann D, Riad A, Lettau O, Roks A, Savvatis K, Becher PM, Escher F, Danser AHJ, Schultheiss HP, Tschöpe C. Renin inhibition ablates the (P)RR observed by others, raises concern regarding the use of HRP in cardiovascular patients, despite its beneficial effects in diabetic nephropathy and retinopathy. Clearly, a detailed insight into its mechanism of action is warranted, investigating not only whether it acts as a partial agonist, but also establishing to what degree HRP exerts effects that are unrelated to the (P)RR. Importantly in this regard, Leckie and Bottrell were unable to demonstrate a specific binding site for HRP on human endothelial cells,\textsuperscript{38} and HRP binding also occurred in cells not expressing the (P)RR.\textsuperscript{39}


Handle Region Peptide Counteracts the Beneficial Effects of the Renin Inhibitor Aliskiren in Spontaneously Hypertensive Rats

Joep H.M. van Esch, Richard van Veghel, Ingrid M. Garrelds, Frank Leijten, Angelique M. Bouhuizen and A.H. Jan Danser

Hypertension. published online February 14, 2011; Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/early/2011/02/14/HYPERTENSIONAHA.110.169060

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at: http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at: http://hyper.ahajournals.org//subscriptions/