Deterioration of kidney function by the (pro)renin receptor blocker handle region peptide in aliskiren-treated diabetic transgenic (mRen2)27 rats

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HYPERTENSIVE PATIENTS WITH DIABETES exhibit an increased risk for cardiovascular complications such as nephropathy, stroke, and heart failure. The renin-angiotensin system (RAS) is believed to modulate the underlying structural and functional changes in the kidney and heart (29, 33), thereby explaining the beneficial effects of RAS blockers in this condition. Elevated levels of prorenin, the precursor of renin, are an early indicator of nephropathy in diabetes (9, 32). Prorenin has been speculated to contribute to ANG generation in the kidney via binding to the so-called (pro)renin receptor ([P]RR). Indeed, (P)RR-bound prorenin displays ANG I-generating activity (1, 37). However, it also stimulates (P)RR-mediated signal transduction in an ANG-independent manner, resulting in the activation of ERK1/2, cyclooxygenase (COX)-2, and fibrotic pathways (2, 37). The latter includes enhanced transforming growth factor (TGF)-β synthesis, plasminogen activator inhibitor (PAI)-1 release, and the upregulation of fibronectin and collagens (2, 18, 19, 37). In agreement with this concept, ubiquitous expression of the human (P)RR in rats leads to proteinuria, glomerulosclerosis, and nephropathy, which could be reversed by the putative (P)RR blocker handle region peptide (HRP) (25). Beneficial renal effects of HRP were also observed in ANG II type 1a receptor (AT1R)-deficient mice, suggesting that they are not solely due to interference with the RAS. However, the capacity of HRP to block (P)RR is controversial (34), and recent studies (28, 38, 40) in knockout animals have suggested that (P)RR deletion in cardiomyocytes or podocytes is actually lethal. Thus, a relevant question is to what degree HRP should still be used, e.g., on top of RAS blockade in diabetic patients with nephropathy and heart failure. This is of particular importance now that the combination of two or more RAS blockers is no longer advocated in diabetic patients, since the side effect profile (hypotension and hyperkalemia) of this approach outweighs the beneficial effects (7, 39).

In the present study, we therefore set out to study the effects of HRP on top of renin inhibition (with aliskiren) in a well-established high-prorenin model, the TGR(mRen2)27 (Ren2) rat, which overexpresses the mouse Ren2 gene (36) and also displays elevated (P)RR levels (5). Aliskiren is renoprotective in this model, and its effects are comparable with those observed during AT1R blockade or ANG-converting enzyme inhibition, despite the nonequivalent blood pressure-lowering effects of these three types of RAS blockers (27, 45). Cardioprotective effects of aliskiren have also been observed in diabetic rodents (10, 47).

Rats were made diabetic with streptozotocin (STZ) and treated for 3 wk with aliskiren and/or HRP. We used a dose of HRP that has been applied before in several rodent studies (12, 22, 23, 25). We reasoned that, if anywhere, the beneficial effects of this putative (P)RR blocker on the kidney and heart should be observed in this high-prorenin, high-(P)RR model.

METHODS

Animal Experiments

Homozygous Ren2 rats (400–500 g, a kind gift from Dr. M. Bader, Berlin, Germany) were crossed with Sprague-Dawley rats (Harlan, Boxmeer, The Netherlands) to generate heterozygous Ren2 rats. Heterozygous rats were subsequently used in all experiments, since...
Mean arterial pressure on day 21, mmHg†

Body weight, kg 0.562 ± 0.02
Heart weight, g 2.16 ± 0.06
Heart weight/body weight, g/kg 3.9 ± 0.10
Kidney weight, g 1.48 ± 0.06
Kidney weight/body weight, g/kg 2.7 ± 0.12
Blood glucose, mM‡

Urine
Creatinine, μmol/day 128 ± 4
Endotoxin-1, pg/day 3.6 ± 0.7
Creatinine clearance, ml/min

Plasma
Creatinine, mmol/ml 32.6 ± 1.6
Endotoxin-1, pg/ml 9.1 ± 4
Plasminogen activator inhibitor-1, ng/ml 3.2 ± 0.5
TG-β1, pg/ml 423 ± 99

Data are means ± SE; n = 7–8 rats/group. Non-DM, nondiabetic; DM, diabetic; HRP, handle region peptide; ND, not determined. Creatinine clearance in non-DM rats could not be calculated since plasma and urine creatinine levels in this group were determined on day 21 and day –14, respectively, i.e., not on the same day. *P < 0.05 vs. non-DM rats; †P < 0.05 vs. vehicle; ‡Data are from Batenburg et al. (3).
Biochemical Measurements

ET-1 was assessed by chemiluminescent ELISA (QuantiGlo, R&D Systems), albumin by enzyme immunoassay (Spi-Bio, Montigny-Le-Bretonneux, France), and aldosterone by radioimmunoassay (Coat-a-Count, Siemens Medical Solutions Diagnostics, Los Angeles, CA), TGF-β1, cystatin C (Quantikine, R&D systems), and PAI-1 (Zymutest, Tebu-Bio, Le Perray-en-Yvelines, France) were measured by ELISA. Creatinine, K⁺, Na⁺, and total protein were measured at the clinical chemical laboratory of the Erasmus MC. ANG I and ANG II were measured by radioimmunoassay, after SepPak extraction and reverse-phase HPLC separation as previously described (6, 8).

Histology

After fixation, kidney and heart sections were dehydrated and paraffin embedded. Gomori silver staining was applied to sections (5 μm) of the left ventricle of the heart to visualize individual cardiomyocytes. Sirius red staining was applied to visualize collagen as a measure of cardiac fibrosis. Cardiomyocyte size and the amount of collagen were measured using Qwin (Leica).

Transversely sliced kidney sections (deparaffinized, 2 μm) were stained with periodic acid-Schiff to localize kidney damage. Cardiomyocyte size and the presence of focal segmental glomerulosclerosis was assessed in all glomeruli of one kidney section per animal with a mean of 181 ± 4 glomeruli per section. All sections were semiquantitatively scored by a renal pathologist in a blinded manner. Renal scarring of all glomeruli was scored on an arbitrary scale from 0 to 4, where grade 0 (n₀) indicates no glomerulosclerosis, grade 1 (n₁) indicates <25% of sclerosis, grade 2 (n₂) indicates 25–50% of sclerosis, grade 3 (n₃) indicates 50–75% of sclerosis, and grade 4 (n₄) indicates >75% of sclerosis.

The presence of focal segmental glomerulosclerosis was assessed in all glomeruli of one kidney section per animal with a mean of 181 ± 4 glomeruli per section. All sections were semiquantitatively scored by a renal pathologist in a blinded manner. Renal scarring of all glomeruli was scored on an arbitrary scale from 0 to 4, where grade 0 (n₀) indicates no glomerulosclerosis, grade 1 (n₁) indicates <25% of sclerosis, grade 2 (n₂) indicates 25–50% of sclerosis, grade 3 (n₃) indicates 50–75% of sclerosis, and grade 4 (n₄) indicates >75% of sclerosis.

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3 (n₁) indicates 50–75% of sclerosis, and grade 4 (n₄) indicates >75% of sclerosis per glomerulus. Hereafter, the individual glomerulocytosclerosis index (GSI) was calculated for each rat with the following formula: [(1 × n₁) + (2 × n₂) + (3 × n₃) + (4 × n₄)] / (n₀ + n₁ + n₂ + n₃ + n₄). Furthermore, 10 images of each kidney section (×100 magnification) were analyzed for arterial hyalinosis, intima fibrosis, and media hypertrophy as well as tubular atrophy, interstitial fibrosis, and renal inflammation according to the Banff classification (35). Each parameter was graded in 10 fields with a score of 0–3, in which 0 indicates no changes in pathology, grade 1 indicates <25% of change, grade 2 indicates 25–50% of change, and grade 3 indicates >50% of change in affected tissue. From these data, the tubulointerstitial score was calculated by dividing the combined score of tubular atrophy, interstitial fibrosis, and renal inflammation with the number of parameters.

Quantitative Real-Time RT-PCR

Total RNA was isolated from snap-frozen rat kidneys and hearts using TRIzol (Life Technologies) and reverse transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Venlo, The Netherlands). The resulting cDNA was amplified for 40 cycles (denaturation at 95°C for 10 min, thermal cycling at 95°C for 15 s, and annealing/extension at 60°C for 1 min) with a Step-One cycler (NYSE, Life Technologies) using SYBR Green PCR Master Mix (Life Technologies). The intron-spanning oligonucleotide primers for quantitative PCR were designed with Primer-BLAST (National Center for Biotechnology Information; Table 1). The comparative cycle time method (ΔΔCₜ, where Cₜ is threshold cycle) was used for the relative quantification of gene expression using the geometric mean of the housekeeping genes hypoxanthine phosphoribosyl transferase-1, AJP-Renal Physiol • doi:10.1152/ajprenal.00010.2014 • www.ajprenal.org
β₂-microglobulin, and β-actin for normalization. In cardiac left ventricular tissue, gene expression of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), β-myosin heavy chain (β-MHC), (P)RR, AT₁R, collagen-1, TGF-β₁, TNF-α, NF-κB, COX-2, and neutrophil gelatinase-associated lipocalin (NGAL; a marker of tubular damage) was measured in the kidney medulla and cortex.

**Statistical Analysis**

Statistical analysis was performed using an unpaired t-test after one-way ANOVA with a Bonferroni posttest confirmed differences between groups. Data are given as means ± SE. P values of <0.05 were considered significant.

**RESULTS**

**Animal Characteristics**

Hemodynamic data have been previously reported (3). In brief, diabetic Ren2 rats were severely hypertensive, and a 3-wk treatment with aliskiren (but not vehicle) lowered blood pressure (Table 2). The effect on blood pressure was unaltered with the changes in urinary volume, although HRP did not prevent the effect of aliskiren on albumin (Fig. 1).

DM decreased plasma creatinine, and this was unaffected by aliskiren treatment. HRP, when given on top of aliskiren, normalized plasma creatinine. No significant changes in urinary creatinine or creatinine clearance were observed after DM induction with or without treatment (Table 2). Likewise, there was no change in plasma cystatin C, indicating no change in the glomerular filtration rate due to 5 wk of DM with or without treatment (Fig. 3).

DM marginally decreased plasma renin activity [P = not significant (NS)], and this was accompanied by a significant decrease in ANG levels in the heart and kidney (Fig. 2). Aliskiren further reduced these levels in the kidney, both with and without HRP. Plasma aldosterone levels were also decreased in diabetic Ren2 rats, and this resulted in increased natriuresis and a (nonsignificant) increase in plasma K⁺ levels (Fig. 3). Remarkably, despite the reduction in plasma aldosterone, urinary aldosterone excretion rose eightfold. Aliskiren

**Biochemical Measurements**

STZ-induced DM increased blood glucose in Ren2 rats approximately fourfold, and this was unaffected by treatment (Table 2). DM increased urinary volume time dependently. It was up ≈4-fold after 2 wk and after an additional 3 wk (during treatment with vehicle) ≈13-fold (Fig. 1). Aliskiren prevented this rise in diuresis, most likely due to its effects on blood pressure, whereas HRP negated the protective effects of aliskiren. Changes in urinary protein and albumin ran in parallel with the changes in urinary volume, although HRP did not prevent the effect of aliskiren on albumin (Fig. 1).

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**Fig. 4.** Gene expression analysis of rat renin (A), (pro)renin receptor [(P)RR; B], ANG II type 1 (AT₁) receptor (C), collagen-1 (D), transforming growth factor (TGF)-β₁ (E), cyclooxygenase (COX)-2 (F), and neutrophil gelatinase-associated lipocalin (NGAL; G) in the kidney medulla and cortex from non-DM and DM Ren2 rats, with the latter treated for 3 wk with vehicle, aliskiren, or aliskiren + HRP. Data are means ± SE; n = 5–9. Values are expressed as fold changes versus non-DM. *P < 0.05 vs. non-DM; $P < 0.05 vs. vehicle.
with or without HRP greatly reduced the latter and yet non-significantly increased plasma aldosterone. As a result, natriuresis tended to diminish ($P = \text{NS}$). Of interest, HRP on top of aliskiren further increased plasma K$^+$. Plasma levels of TGF-$\beta_1$ and PAI-1 were not affected by diabetes or treatment with aliskiren. However, HRP on top of aliskiren increased plasma PAI-1 by 50% compared with vehicle- or aliskiren-treated rats without affecting TGF-$\beta_1$.

Fig. 5. Top: representative images of periodic acid-Schiff-stained kidney sections (a and b) and $\alpha$-smooth muscle actin-stained interlobar arteries (c). Bottom: glomerular volume (A), glomerulosclerosis index (GSI; B), tubulointerstitial score (TIS; C), interlobar arterial lumen diameter (D), wall thickness (E), and wall-to-lumen ratio (F) in kidney sections from non-DM and DM Ren2 rats, with the latter treated for 3 wk with vehicle, aliskiren, or aliskiren + HRP. Data are means $\pm$ SE; $n = 7–8$. *$P < 0.05$ vs. non-DM.
Plasma and urinary ET-1 levels were unaffected by DM or treatment (Table 2).

The Kidney

**Gene expression.** DM did not alter rat renin, AT₁R, COX-2, or Ngal expression in the medulla or cortex (Fig. 4). It increased (P)RR and collagen-1 expression in the medulla and, to a lesser degree (P = NS), in the cortex. DM decreased TGF-β₁ expression in the cortex, whereas it induced a nonsignificant increase in TGF-β₁ expression in the medulla. Similar changes were observed for TNF-α and NF-κB, but these were not significant (data not shown). Aliskiren did not alter DM-induced changes in TGF-β₁ and collagen-1 and increased cortical rat renin expression. A similar increase in renin expression, albeit nonsignificant, was observed in the medulla. Aliskiren diminished cortical (P)RR and AT₁R expression without affecting these parameters in the medulla. Aliskiren tended to increase cortical COX-2 expression (P = NS). Changes by aliskiren were unaltered in the presence of HRP except for the increase in COX-2, which became significant after the addition of HRP.

**Histology.** DM did not alter tubulointerstitial score, glomerular volume, interlobar arterial lumen diameter, and wall thickness (nor the ratio of the latter two) and nonsignificantly decreased GSI (Fig. 5). Arterial hyalinose, intima fibrosis, and media hypertrophy were not observed. Aliskiren reduced GSI without affecting any of the other parameters. HRP, when given on top of aliskiren, did not alter its effects on GSI but tended to increase glomerular volume and lumen diameter, with the latter resulting in a decrease in the lumen diameter-to-wall thickness ratio. However, none of these changes were significant.

The Heart

**Gene expression.** DM did not affect cardiac ANP, BNP, and (P)RR expression and increased β-MHC and AT₁R expression (Fig. 6). Aliskiren with HRP, but not aliskiren alone, normalized the latter. Drug treatment did not affect (P)RR or β-MHC expression. Aliskiren, with or without HRP, reduced cardiac BNP expression, and similar trends were observed for cardiac ANP expression, although the changes were significant only during combination treatment.

**Histology.** DM increased cardiac collagen content without altering myocyte size. Aliskiren did not affect these changes, whereas aliskiren with HRP further increased collagen content and marginally diminished myocyte size (Fig. 7).

**DISCUSSION**

This study shows that HRP counteracts the favorable effects of aliskiren on early renal damage in diabetic Ren2 rats. In
agreement with previous studies (3, 26), the hypertensive Ren2 rat, when made diabetic with STZ, displayed mild glomerulosclerosis, accompanied by albuminuria, proteinuria, and diuresis. A 3-wk treatment with aliskiren improved these parameters, whereas the addition of HRP on top of aliskiren negated the protective effects of aliskiren on the latter two. HRP also induced hyperkalemia and increased plasma PAI-1, renal COX-2, and cardiac collagen content. This argues against the application of HRP in combination with aliskiren in diabetic patients.

Plasma creatinine decreased after the induction of DM, most likely reflecting the weight (and muscle) loss occurring in these animals. There were no changes in cystatin C or renal Ngal expression, suggesting that indeed the renal damage in our DM animals was at an early stage, not yet resulting in alterations in glomerular filtration or tubular damage. Of interest, aliskiren alone did not alter these parameters, whereas HRP on top of aliskiren increased plasma creatinine and tended to increase \( (P = \text{NS}) \) cortical Ngal expression, again suggesting that HRP, if anything, worsened renal function when combined with aliskiren.

DM reduced plasma, cardiac, and renal RAS activity in the Ren2 rat, although only the reduction in tissue was significant. The reduction in plasma renin activity was modest, in full agreement with the consequence of diabetes in humans (15). Along with this RAS suppression, plasma aldosterone decreased by almost 50%. Not surprisingly, this resulted in natriuresis and a (nonsignificant) rise in plasma \( K^+ \). Yet, urinary aldosterone excretion increased eightfold. This is suggestive for a net rise in adrenal aldosterone production, most likely to compensate the loss of aldosterone via urine (\( \approx 1.5 \text{ ng/day} \)). Of interest, aliskiren greatly diminished the urinary aldosterone loss, reflecting a reduction in aldosterone production, although plasma aldosterone, if anything, tended to go up, thereby counteracting the above effects on natriuresis and hyperkalemia. The effects of aliskiren on aldosterone and natriuresis were unaltered by HRP. However, it greatly elevated plasma \( K^+ \). Since this occurred independently of
changes in aldosterone, it might be the consequence of direct effects of HRP, e.g., via (P)RR (31).

The aliskiren-induced reduction in renal ANG content, together with the reduction of cortical AT1R expression, probably underlies the beneficial effect of renin inhibition in the kidney. Aliskiren-induced AT1R suppression has been previously reported, both in the kidney and other organs (11, 30, 43). At 5 wk after STZ injection, we observed modest regional changes in renal TGF-β1, TNF-α, NF-κB, and collagen-1 expression, although no fibrosis or inflammation could be detected. It is therefore not surprising that aliskiren did not significantly affect these parameters in the kidney. Such effects have been observed before, but this required a longer duration of DM (10 wk) and aliskiren treatment starting at the moment of STZ injection (11). HRP did not alter the effect of aliskiren on TGF-β1 but unexpectedly increased the levels of PAI-1. These observations contrast with the idea that HRP prevents (pro)renin-(P)RR interactions, thereby blocking the rise in PAI-1 that result from such (P)RR stimulation, at least in vitro (19, 48). Possibly, the increase in renal (rat) renin expression after aliskiren was too modest to increase PAI-1. In addition, aliskiren suppressed (P)RR expression. Recently, HRP has been reported to act as a partial agonist of (P)RR (31, 46). Thus, its stimulatory effects on PAI-1 on top of aliskiren might also be the consequence of direct (P)RR stimulation.

Hyperglycemia elevates (P)RR expression in rat mesangial cells via PKC, ERK1/2, and JNK (17), and this has been suggested to facilitate ANG II generation and AT1R-dependent COX-2 induction (13). Ubiquitous overexpression of human (P)RR in the rat also resulted in COX-2 upregulation (25). Simultaneously, COX-2 inhibition reduced the glucose-induced (P)RR upregulation, suggesting that COX-2 itself upregulates (P)RR (16). Our study confirms renal (P)RR upregulation in diabetic Ren2 rats. However, significant COX-2 upregulation was only seen after concomitant HRP administration, even in the face of aliskiren-induced (P)RR suppression. This suggests that (P)RR upregulation per se is insufficient to increase COX-2 and requires additional (P)RR stimulation, either by renin, HRP, or their combination. COX-2 elevation has been previously reported in the macula densa after renin upregulation due to salt restriction (14). Such COX-2 upregulation has detrimental effects, for instance, COX-2-generated endothelium-derived contractile factors in diabetic Ren2 rats, thereby inducing vascular dysfunction (3), and the COX-2 upregulation in human (P)RR-overexpressing rats was accompanied by proteinuria and glomerulosclerosis (25).

Natriuretic peptides are released by the hypertrophied heart, and their levels are elevated in patients with heart failure (4) and in homozygous Ren2 rats (44). Aliskiren reduced cardiac ANP and BNP expression in the diabetic, heterozygous Ren2 rats of the present study, most likely due to its blood pressure-lowering effect. Yet, aliskiren did not affect cardiac hypertrophy, β-MHC expression, or myocyte size. These effects were unaltered by HRP. Moreover, no changes occurred in cardiac (P)RR expression, suggesting that (P)RR upregulation by hyperglycemia is not a uniform phenomenon. Although aliskiren with or without HRP did not significantly reduce cardiac ANG content, HRP combined with aliskiren did suppress cardiac AT1R expression, suggesting that this combination may have reduced the consequences of ANG II-AT1R interactions. However, this did not result in a reduction of cardiac fibrosis, possibly because the degree of fibrosis in our model was still modest. Remarkably, however, cardiac fibrosis increased significantly after cotreatment of aliskiren with HRP. This contrasts with a previous study (21) showing antifibrotic effects of HRP in stroke-prone spontaneously hypertensive rats and once again illustrates the partial agonistic capacities of HRP.

In conclusion, renin inhibition improves renal function in diabetic Ren2 rats with early kidney damage, and (P)RR blockade with HRP not only counteracted this effect in a RAS-independent manner but also increased K+-PAI-1, renal COX-2, and cardiac fibrosis. This contrasts with the beneficial cardiac and renal effects of HRP observed in various models (20, 22, 24) but agrees with the deleterious effects of (P)RR knockout in the heart and kidney (28, 40). A uniform explanation might be that HRP acts as a partial agonist (43, 46). Nevertheless, given these controversial findings, it seems that, at this stage, HRP should not be considered as add-on drug in diabetics treated with a RAS inhibitor. Furthermore, given the uncertainty whether HRP is a (P)RR blocker or not, future studies should carefully examine the exact function of (P)RR in diabetes, e.g., making use of (inducible) renal cell-specific knockout models, to define its role as a treatment target.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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