Abstract—The aim of this study was to explore the effects of the renin inhibitor aliskiren in streptozotocin-diabetic TG(mRen-2)27 rats. Furthermore, we investigated in vitro the effect of aliskiren on the interactions between renin and the (pro)renin receptor and between aliskiren and prorenin. Aliskiren distributed extensively to the kidneys of normotensive (non)diabetic rats, localizing in the glomeruli and vessel walls after 2 hours exposure. In diabetic TG(mRen-2)27 rats, aliskiren (10 or 30 mg/kg per day, 10 weeks) lowered blood pressure, prevented albuminuria, and suppressed renal growth (the β growth factor) and collagen I expression versus vehicle. Aliskiren reduced (pro)renin receptor expression in glomeruli, tubules, and cortical vessels compared to vehicle (in situ hybridization). In human mesangial cells, aliskiren (0.1 μmol/L to 10 μmol/L) did not inhibit binding of [125I]-renin to the (pro)renin receptor, nor did it alter the activation of extracellular signal-regulated kinase 1/2 by renin (20 nmol/L) preincubated with aliskiren (100 nmol/L) or affect gene expression of the (pro)renin receptor. Evidence was obtained that aliskiren binds to the active site of prorenin. The above results demonstrate the antihypertensive and renoprotective effects of aliskiren in experimental diabetic nephropathy. The evidence that aliskiren can reduce in vivo gene expression for the (pro)renin receptor and that it may block prorenin-induced angiotensin generation supports the need for additional work to reveal the mechanism of the observed renoprotection by this renin inhibitor. (Hypertension. 2008;52:130-136.)

Key Words: aliskiren ■ renin inhibitor ■ TG(mRen-2)rat ■ diabetic nephropathy ■ (pro)renin receptor

A central role for the renin-angiotensin-aldosterone system (RAAS) in the pathogenesis of diabetic nephropathy (DN) is widely accepted, based largely on the attenuation of DN by angiotensin (Ang) converting enzyme inhibitors (ACEi)1 and Ang II receptor blockers (ARB).2 However, these agents do not halt renal decline, possibly because of insufficient suppression of the intrarenal RAAS. Theoretically, agents that more effectively suppress the RAAS should confer improved tissue protection over current treatments for DN. Renin inhibitors, by acting at the point of activation of the RAAS cascade, may represent such agents. Aliskiren is a potent inhibitor of human renin; it lowers blood pressure (BP) in patients with mild-moderate hypertension3,4 and shows cardiorenal protection in hypertensive double transgenic rats expressing human genes for renin and angiotensinogen.5

The cloning of a functional receptor for renin and prorenin [(P)RR]6 suggests that renin and prorenin [collectively, (pro)renin] may exert direct (receptor-mediated, Ang II–independent) tissue-damaging effects by increasing the expression of profibrotic pathways7 and molecules such as TGF-beta, (TGF-β).8 Moreover, prorenin, the inactive proenzyme form of renin, may contribute to tissue damage9,10 via binding to and activation of the (P)RR. On binding to the (P)RR, prorenin undergoes nonproteolytic activation,6,11 a conformational change that exposes its active site without removal of the prosegment.12,13 This permits cell surface generation of Ang-I,6,11 and via subsequently formed Ang II, may contribute to tissue damage6,10 by binding to and activation of the (P)RR. On binding to the (P)RR, prorenin undergoes nonproteolytic activation,6,11 a conformational change that exposes its active site without removal of the prosegment.12,13 This permits cell surface generation of Ang-I,6,11 and via subsequently formed Ang II, may contribute to the development of DN.14,15 Additionally, the (P)RR may amplify renin-induced Ang II–dependent effects; renin bound to the (P)RR gains ~5 fold enhanced catalytic activity versus soluble renin.6

TG(mRen-2)27 rats express the mouse ren-2 gene and become hypertensive.16 When rendered diabetic with streptozotocin (STZ), they develop renal damage considered analogous to that seen in human DN.17 Aliskiren shows antihypertensive and renoprotective effects in this model.18

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Here we explored potential renoprotective mechanisms of aliskiren in diabetic TG(mRen-2)27 rats. Thus, we confirmed the antihypertensive and antialbuminuric effects of aliskiren, determined its renophilic nature, and studied whether this renin inhibitor can (1) bind to the active site of prorenin in vitro; (2) interfere with the binding of renin to the (P)RR in vitro; and (3) alter the gene expression of renal (P)RR, TGF-β1, and collagen I in vivo.

Materials and Methods
For details of the methods and statistical procedures please see http://hyper.ahajournals.org.

In Vitro Studies

Determination of IC₅₀ of Aliskiren Against Mouse Renin
The IC₅₀ for aliskiren against mouse submaxillary gland renin was determined by measuring the rate of cleavage of the fluorescence-quenched substrate RE(EDANS)IFHPHLVIHTK(Dabcyl)R (Biodynathm GmbH).

Ability of Aliskiren to Bind to Active Site of Prorenin
Prorenin exists in 2 conformations: a catalytically inactive form, where the prosegment covers the active site (“closed”), and an “open” conformation where the prosegment does not cover the active site, permitting access to substrate. Open prorenin is catalytically active and recognizable by antibodies directed against epitopes in the active site. Thus, aliskiren-enhanced antibody binding to prorenin would indicate binding of aliskiren to the active site of prorenin, thereby preventing the prosegment from returning to its “closed” position.

We applied an immunoradiometric assay to determine the ability of increasing aliskiren concentrations to bind to prorenin from a pool of human plasma.

Effect of Aliskiren on Binding of Renin to (P)RR, on ERK Activation, and Gene Expression of (P)RR in Mesangial Cells
For each experiment, human mesangial cells were exposed for 4 hours at 19°C (prevents internalization) ± cold renin (100 nmol/L) to 125I-renin (1 nmol/L), or to 125I-renin that was untreated or preincubated with aliskiren (0.1 µmol/L to 10 µmol/L). Specific binding of renin was determined by subtracting nonspecific binding from total renin binding.

Phosphorylation of ERK 1/2 was studied by immunoblotting in cells exposed to renin (20 nmol/L) ± preincubation for 30 minutes at 37°C with excess aliskiren (100 nmol/L).

To determine whether aliskiren could alter gene expression of (P)RR in vitro, mesangial cells were incubated with 1 µmol/L aliskiren for 24 hours, mRNA was extracted, and real-time (quantitative) RT-PCR was performed to assess gene expression of (P)RR.

In Vivo Studies
Animal procedures conformed to guidelines of the Novartis Animal Care and Use Committee.

Renal Partitioning and Localization of Aliskiren in Rats
Streptozotocin-induced diabetic male Sprague-Dawley (S-D) rats and normoglycemic controls were used. Aliskiren treatment (3, 10 mg/kg day by subcutaneously implanted osmotic Alzet minipumps) commenced 1 day after induction of diabetes. Two weeks later animals were euthanized and kidneys were removed for measurement of aliskiren levels.

To identify the potential renal compartments accessible to aliskiren, 2 normal male Hanover Wistar rats received a single i.v. dose of 14C-aliskiren (10 mg/kg). Two hours later the rats were euthanized and the kidneys were removed and snap frozen. Subsequently, 10-µm-thick cryosections were processed for autoradiography.
rats (10 mg/kg per day) and 29.5 ± 4.6 ng/mL and 128 ± 22.7 ng/mL in diabetic rats (3, 10 mg/kg per day, respectively). The mean kidney/plasma concentration ratio of aliskiren in rats treated with the compound for 2 weeks was 45.7 for nondiabetic rats (10 mg/kg per day) and was 30.8 and 63.7 for diabetic rats (3, 10 mg/kg per day, respectively; Figure 3A), indicating extensive partitioning of aliskiren to the kidneys. In rats treated with 10 mg/kg per day of aliskiren, renal content of aliskiren was lower in diabetic versus nondiabetic rats, but plasma levels of aliskiren were similar.

By light microscopy, 100% of glomeruli on each renal section showed localization of autoradiographic grains, indicating the presence of aliskiren (Figure 3B). The cell type in which aliskiren was localized could not be determined from the 10-µm-thick frozen sections used in our study. Regardless, the pattern did not resemble an exclusively luminal distribution. Extensive labeling was also found in the arterial wall of the small cortical vessels in the kidney (Figure 3C).

Effect of Aliskiren in Diabetic TG(mRen-2)27 Rats

IC50 of Aliskiren Against Mouse Renin
The IC50 for aliskiren against mouse renin was determined to be 4.5 nmol/L, confirming that aliskiren effectively inhibits the enzymatic activity of mouse renin.

Body Weight and Blood Glucose
At the end of the study, diabetic TG(mRen-2)27 rats showed no statistically significant differences between groups in body weight or gain in body weight. The intended level of glucose control (400 to 525 mg/dL) was achieved during the study. Areas under the blood glucose versus time curves were not significantly different between groups (Table S1).

Effect of Aliskiren on BP, Heart Rate, Albuminuria
There was a trend for a mild and gradual decline in mean arterial pressure (MAP) in vehicle-treated diabetic TG(mRen-2)27 rats during the study (Figure 4A). In contrast,
initiation of treatment with aliskiren resulted in a prompt and sustained reduction in MAP. Five days after starting treatment with aliskiren, average MAP was reduced by 36±4 and 51±3 mmHg by aliskiren 10 and 30 mg/kg per day, respectively. The antihypertensive effect of aliskiren appeared to be dose-related for the initial 4 weeks of treatment, after which an additional BP lowering effect was noted in both groups.

Heart rates were not significantly affected by aliskiren treatment (data not shown).

At the start of the study albuminuria did not differ significantly between the groups. The progressive albuminuria seen in vehicle-treated TG(mRen-2)27 rats did not develop in aliskiren-treated TG(mRen-2)27 rats (Figure 4B).

**Effect of Aliskiren on Renal Cortical Gene Expression of TGF-β, Collagen I, III, IV, and (P)RR**

At the end of the study, renal cortical gene expression of TGF-β was significantly suppressed in aliskiren- versus vehicle-treated diabetic TG(mRen-2)27 rats (Figure 5A), with the 10 mg/kg per day group tending to be slightly but not significantly greater than in the 30 mg/kg per day group. Renal gene expression of collagen I was significantly reduced by treatment of diabetic TG(mRen-2)27 rats with both aliskiren doses (Figure 5B). Expression of collagens III and IV was not significantly altered by aliskiren treatment compared to vehicle-treated diabetic TG(mRen-2)27 rats (data not shown).

In situ hybridized renal sections from vehicle-treated diabetic TG(mRen-2)27 rats showed prominent labeling for (P)RR in glomeruli and tubules and less in renal arteries (Figure 6A, 6C, 6E). However, in aliskiren-treated TG(mRen-2)27 rats, expression of (P)RR in these renal compartments was markedly suppressed compared to vehicle-treated controls (Figure 6B, 6D, 6F).

**Effect of Aliskiren on Gene Expression of Renal Rat Renin**

At the end of the study gene expression of endogenous rat renin was significantly and dose-dependently increased above vehicle-treated rats (Figure 7), indicating aliskiren-induced RAAS blockade.

**Discussion**

The main findings in this study were that in a model of hypertensive diabetic renal damage, renin inhibition with aliskiren lowered BP, prevented albuminuria, and suppressed renal gene expression of the (P)RR. Moreover, aliskiren displayed the potential to inhibit prorenin. Thus, aliskiren may exert multiple renoprotective mechanisms.

In TG(mRen-2)27 rats, mouse renin contributes to the elevated BP.16,23 Thus, the observed inhibitory potency of aliskiren against mouse renin (IC₅₀ 4.5 nmol/L) suggests that aliskiren lowered BP in TG(mRen-2)27 rats by inhibiting mouse renin. The increased renal gene expression of (endogenous) rat renin in these animals, indicating RAAS blockade, further supports this assertion.

The extensive partitioning of aliskiren to the kidneys suggests a renoprotective effect via inhibiting the intrarenal RAAS, in addition to the antihypertensive effect of the drug. Moreover, the localization of autoradiographic grains in glomeruli and the vascular wall, indicating the presence of aliskiren, suggests the potential for local renin inhibition within these structures. Longer exposures to aliskiren may result in localization of the drug in other renal compartments such as the tubulo-interstitium. Importantly, the presence of aliskiren in the vessel wall suggests that aliskiren may enter the juxtaglomerular cells of the afferent arteriole, the site of renin synthesis, opening the possibility that aliskiren might inhibit forming (or formed, but still intracellular) renin even before its release from juxtaglomerular cells. Indeed, blockade of intracellular renin by aliskiren has been reported in cultured cardiomyocytes.24

Recently Kelly et al18 reported in diabetic TG(mRen-2)27 rats that despite not lowering BP to the same extent as the ACEi perindopril, the administered dose of aliskiren attenuated tubulo-interstitial fibrosis to a greater extent than the ACEi and reduced albuminuria and glomerulosclerosis to similar levels as the latter agent.

In our study, treatment of diabetic TG(mRen-2)27 rats with aliskiren also prevented, for the entire study, the development
of albuminuria that was seen in vehicle-treated controls. These findings are relevant because albuminuria is considered a marker for risk of renal decline.25

The extent to which the antialbuminuric effect of aliskiren was attributable to its antihypertensive effect per se, compared to a BP-independent effect, was not addressed in this study.

TGF-β1 in conjunction with Ang II plays a central role in renal fibrosis.26 Our data indicate that aliskiren suppressed the renal gene expression of TGF-β1 in vivo and thereby may have potential to inhibit TGF-β1–mediated pathways toward renal fibrosis. Indeed, in our study aliskiren reduced renal collagen I gene expression. The lack of corresponding effects on collagen III and IV may reflect a differential level of synthetic activity of these collagens at this stage in the experimental model.

The recent cloning of the (P)RR6 and its implication in cardio-renal disease14,15,21 prompted us to explore a possible relationship between renoprotection of aliskiren and the function of this receptor. Our in vitro results indicate that aliskiren, as with remikiren,7 did not induce a conformational change in the renin molecule extensive enough to interfere with a renin–(P)RR interaction. Thus, aliskiren is not a (P)RR antagonist, and the aliskiren-induced reduction in BP, renal TGF-β1, and collagen I expression, and the prevention of albuminuria are unlikely to result from inhibition of renin binding to its receptor.

Despite the above in vitro findings, the observations by in situ hybridization indicate an aliskiren-induced suppression of gene expression of (P)RR in vivo. Incubation of aliskiren with mesangial cells did not alter gene expression of (P)RR in these cells, arguing against a direct effect of

Figure 5. A and B, Aliskiren treatment suppresses renal cortical gene expression for TGF-β1 and collagen I in diabetic TG(mRen-2)27 rats. TG(mRen-2)27 rats were treated with vehicle or aliskiren for 10 weeks. Renal cortical TGF-β1 (A) and collagen I (B) gene expressions are expressed as % vehicle control after standardization against GAPDH.

Figure 6. Aliskiren treatment suppresses gene expression of (P)RR in diabetic TG(mRen-2)27 rats; in situ hybridization. Expression of (P)RR was prominent in glomeruli (A, B; 40×), tubules (C, D; 16×), and small cortical vessels (E, F; 40×) in vehicle-treated rats (A, C, E), but was reduced in aliskiren-treated rats (B, D, F; aliskiren 10 mg/kg per day).
aliskiren on (P)RR expression in vivo. This lack of in vitro effect may reflect the absence of physiological mechanisms that may be required for aliskiren to indirectly reduce (P)RR expression in vivo; possibly by (1) reducing renal/plasma Ang II levels, (2) increasing renal/plasma (pro)renin levels, (3) recruiting intracellular promyelocytic zinc finger,27 or simply (4) by lowering BP.

The current in situ hybridization results show a distribution pattern in more renal compartments than initially described in human kidneys.6 However, our results are consistent with a more recent report of tubular expression of the (P)RR gene in human kidneys.28

Huang et al showed that in cultured mesangial cells, stimulation of the (P)RR with renin resulted in TGF-β production8 and extracellular matrix protein synthesis, and that suppressing the (P)RR inhibited the production of these latter factors.29 Our in vivo observations appear to be consistent with those findings. However, we emphasize that in the current study a causal link between the salutary effects induced by aliskiren in TG(mRen-2)27 rats and the observed aliskiren-induced suppression of renal gene expression of the (P)RR is not clearly defined.

Diabetic TG(mRen-2)27 rats develop high levels of circulating prorenin by 4 weeks after STZ-induced diabetes,17 and this prorenin may in part contribute to intrarenal angiotensin generation.14 The current results indicate that (P)RR-mediated activation of prorenin and the consequent gain in Ang II forming ability* may be neutralized by aliskiren, because like other renin inhibitors,12 aliskiren bind to the active site of prorenin. Once bound to “open” prorenin, aliskiren is unlikely to dissociate, because of its high affinity for the active site. Moreover, this binding should block any enzymatic activity gained by (non)-proteolytic activation of the molecule. Consequently, tissue damage related to angiotensin generated by (P)RR-bound, nonproteolytically activated prorenin, or prorenin that becomes proteolytically activated at tissue sites should be reduced by aliskiren.

The present work supports the conclusion that, in diabetic TG(mRen-2)27 rats, aliskiren is an antihypertensive renoprotective agent that does not act by interfering with the function of the (P)RR. Thus, to the extent that the (P)RR may play a role in the pathogenesis of experimental DN,14,15 we speculate that besides lowering systemic BP and blocking the circulating and tissue RAAS, aliskiren may be antifibrotic by the following nonmutually exclusive (P)RR-mediated actions: (1) suppression of (P)RR gene expression may cause less receptor number and dampen intracellular fibrotic pathways induced by (pro)renin, (2) preventing the activation of prorenin in renal tissue, (3) negating the gain in catalytic activity of receptor-bound renin. However, more work is needed to determine whether the (P)RR plays a pathogenic role in DN.

**Perspectives**

Patients treated with RAAS blocking agents, including aliskiren, show high concentrations of plasma renin. If the (P)RR is proven to be important in the pathogenesis of human DN, and if aliskiren suppresses expression of the (P)RR in humans, this action of aliskiren could mitigate the potentially negative consequences of renin-induced activation of the (P)RR.

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