Persistent antihypertensive effect of aliskiren is accompanied by reduced proteinuria and normalization of glomerular area in Ren-2 transgenic rats

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The effects of the human renin inhibitor aliskiren on blood pressure (BP), end-organ damage, proteinuria, and tissue and plasma angiotensin (ANG) II levels in young and adult heterozygous Ren-2 transgenic rats (TGR) were evaluated and compared with the effect of the ANG type 1 (AT1) receptor blocker losartan during treatment and after 12 days after the withdrawal of drug treatments. BP was monitored by telemetry from the age of 32 days on (young rats) and at 100 days (adult rats). Aliskiren (10 mg·kg−1·day−1 in osmotic minipumps) or losartan (5 mg·kg−1·day−1 in drinking water) treatment was applied for 28 days in young rats and for 70 days in adult rats. In young untreated TGR, severe hypertension rapidly evolved. Adult untreated TGR exhibited stable established hypertension. Both aliskiren and losartan fully prevented the development of hypertension and cardiac hypertrophy in young TGR and normalized BP and cardiac hypertrophy in adult TGR. After cessation of aliskiren treatment in both young and adult TGR BP and cardiac hypertrophy were persistently reduced, while after losartan withdrawal BP and cardiac hypertrophy rapidly increased. In adult aliskiren-treated rats proteinuria was significantly reduced compared with losartan (the effect persisting after withdrawal of treatment), and this decrease strongly correlated with normalization of glomerular size in these animals. In conclusion, aliskiren and losartan had similar antihypertensive effects during chronic treatment, but the antihypertensive and organoprotective effects of aliskiren were persistent even after the 12-day washout period. The durable effect on proteinuria can possibly be attributed to the normalization of glomerular morphology.

losartan; hypertension; direct renin inhibition; glomerular size; morphometry; washout period; blood pressure

Aliskiren, the first clinically approved direct renin inhibitor (31), is a human-specific renin inhibitor that has been demonstrated as an effective blood pressure (BP)-lowering compound in animal and human studies (4, 19, 26) with substantial antiproteinuric (14, 26, 28) and cardioprotective (30, 36) activity. Aliskiren is generally well tolerated, and in combination with other antihypertensives, especially in diabetic patients, it has beneficial effects on proteinuria (10, 26). Recently, much attention has been devoted to the role of (pro)renin receptor in prevention of end-organ damage (9, 12) in both experimental and clinical studies.

Models that harbor an extra human [double transgenic rats (2)] or murine [Ren-2 transgenic rats (22)] renin are suitable models for studying the effect of a human renin inhibitor since aliskiren has preferential affinity for human and mouse renin, while that for rat renin is very low. Ren-2 transgenic rats [TGR; official strain name TGR(mRen2-27)] represent a well-established model of ANG II-dependent malignant hypertension.

Aliskiren has been shown to have antihypertensive and cardioprotective effects in Ren-2 TGR (37) and, additionally, to have nephroprotective (14) and antihypertensive (7, 14) effects in diabetic Ren-2 TGR [for review see Feldman (6)]. Moreover, it has been shown that the effect persists after cessation of the treatment (24). However, detailed studies of its mechanism of action are still lacking.

In the present study we investigated the effects of aliskiren on BP, ANG II levels, and end-organ damage in young (prevention protocol) and adult (regression protocol) heterozygous Ren-2 TGR and compared them with the effects of the ANG type 1 (AT1) receptor blocker losartan. Moreover, we were interested to know whether after washout of the drug aliskiren could have additional beneficial effects on functional or physiological parameters over classical renin-angiotensin system (RAS) inhibition.

MATERIALS AND METHODS

The present study was performed in accordance with guidelines and practices established by the Institute for Clinical and Experimental Medicine Animal Care and Use Committee, and protocols were reviewed and approved by this committee (protocol 3/2007). All animals used in the study were housed in facilities accredited by the Czech Association of Laboratory Animal Care.

Animals

Male heterozygous TGR [strain name TGR(mRen2-27)] and normotensive control Hannover Sprague-Dawley rats (HanSD) were housed at 25°C under a 12:12-h light-dark cycle and had free access to chow and water. All animals used in this study were bred at the Center for Experimental Medicine of the Institute for Clinical and Experimental Medicine from stock animals supplied from Max Delbrück Center for Molecular Medicine in Berlin, Germany.
Systolic Blood Pressure and Treatments

In accordance with the recently published recommendation for BP measurement in experimental animals, we employed a radiotelemetry system for direct BP measurements (18). At the age of 32 (young) or 100 (adult) days rats were anesthetized with Zoletil (6.25 mg/kg, Virbac) and Rometar (5 mg/kg, Spofa), and TA11PA-C10 (young) or TA11PA-C40 (adult) radiotelemetric probes (Data Sciences International, St. Paul, MN) were implanted into the aorta (young rats) or into the femoral artery (adult rats). When BP recording was started, either concomitantly (in young rats) or after 1 wk of recovery (in adult rats), losartan (5 mg kg\(^{-1}\)·day\(^{-1}\)) was suspended in drinking water; Sigma Aldrich) or aliskiren (10 mg kg\(^{-1}\)·day\(^{-1}\)) was suspended via osmotic minipumps (type M 2004 for the first 28 days and M 2006 for the next 42 days in the regression study); Alzet, Novartis) was administered. Losartan concentrations were adjusted weekly to maintain the scheduled treatment dose. Measurements of BP [expressed as mean arterial pressure (MAP)] were taken for 10 s every hour in conscious, freely moving rats. After 28 and 70 days of treatment in young and adult rats, respectively, the animals were killed and plasma and tissue ANG II concentrations were determined. The ratio of left ventricle (mg) to tibia length (mm) was used as a measure of cardiac hypertrophy. The treatment period was followed by 12 days without treatment (“washout period”) in experimental and control groups, each with six to nine rats that were killed for determination of the same parameters.

ANG II Determinations

At the end of the experiment animals were decapitated and arterial blood was collected in chilled tubes containing a mixed inhibitor solution (5 mmol/l EDTA, 10 μmol/l pepstatin, 1.25 mmol/l 1,10-phenanthroline, 20 μmol/l enalaprilat). Kidneys were rapidly removed and homogenized in chilled pure methanol. Homogenates were centrifuged at 4°C for 10 min at 3 000 g, plasma was separated, eluted in ethanol, and centrifuged, and both (plasma and homogenates) were evaporated under nitrogen to dryness with the Savant Speed Vac and stored at −80°C until assay. Samples were reconstituted with a phosphate buffer and vortexed thoroughly. An RIA procedure was performed immediately (Euro-Diagnostica, Hamburg, Germany) as described in detail previously (21).

Proteinuria

In weeks 1, 5, 10, and 12 of the experiment, adult rats from the regression protocol were placed in metabolic cages for 24-h urine collection. Urine volume was recorded and protein excretion measured with a commercially available kit (Biolatex).

Histological Examination

At the end of the experiments the left kidney was quickly removed, fixed in 4% buffered formaldehyde, dehydrated, and embedded. Paraffin sections were stained with hematoxylin and eosin and periodic acid Schiff reaction and were examined with a Nicon Eclipse E 600 light microscope. Slides were evaluated in a blinded fashion. As described previously (32), 100 glomeruli per section were randomly selected and the degree of glomerular damage was evaluated with a semiquantitative scoring method: grade 0: normal glomeruli; grade 1: sclerotic area up to 25% or distinct adhesion present between capillary tuft and Bowman’s capsule; grade 2: sclerotic area 25–50% glomeruli; grade 3: sclerotic area 50–75%; grade 4: sclerotic area 75–100%. The glomerulosclerosis index (GSI) was calculated with the following formula: GSI = (1 × n1) + (2 × n2) + (3 × n3) + (4 × n4)n1 + n2 + n3 + n4, where n1 is the number of glomeruli in each grade of glomerulosclerosis.

For electron microscopy small pieces of renal cortex fixed in the same solution were postfixed in 1% osmium tetroxide, dehydrated, and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Philips EM 286/Morgagni transmission electron microscope (FEI, Brno, Czech Republic).

Morphometric Analysis

For morphometric evaluation the cross-sectional area of all consecutive glomerular profiles contained in one kidney section for each animal was measured. Glomerular morphometry was performed with the Nikon NIS-Elements AR 3.1 morphometric program (Nikon, Tokyo, Japan). Five animals in each group were examined in a blinded manner.

Statistical Analysis

Statistical analysis of data was performed with GraphPad Prism software (GraphPad Software, San Diego, CA). ANOVA for repeated measurements, followed by Student-Newman-Keuls test, was performed for the BP analysis within the groups. Statistical comparisons of other results were made by Student’s t-test or one-way ANOVA. Unless noted, values are expressed as means ± SE and n represents the number of animals. A P value <0.05 was considered statistically significant.

RESULTS

MAP and Heart Rate

Prevention protocol. At the beginning of the experiment, MAP did not differ among the groups of young TGR and HanSD (Fig. 1A). BPs of control HanSD were at normotensive values during the entire study. As anticipated, MAP in untreated TGR rose gradually from day 46 onward, with a plateau from day 58 on. Whereas aliskiren treatment had no effect on MAP in control HanSD (data not shown), it prevented the rise in MAP found in untreated young TGR. Interestingly, this effect persisted for 12 days after withdrawal of the treatment. Losartan decreased MAP to the same extent as aliskiren; however, this effect disappeared 2 days after cessation of losartan treatment. From day 61 onward, MAP in losartan-treated TGR was not significantly different from that in untreated TGR.

Regression protocol. At the beginning of the study, MAP did not differ among the three groups of adult TGR but significantly exceeded that of control HanSD (Fig. 1B), which were normotensive during the whole study (data not shown). The decline of MAP was slower with aliskiren than with losartan treatment and declined to levels that were not different from MAP in control HanSD. Similar to young animals, BP remained depressed and normotensive after withdrawal of aliskiren treatment, while in losartan-treated TGR, BP gradually rose throughout the washout period—the difference became significant on day 6 after withdrawal of the treatment but did not reach BP levels of untreated TGR.

Both in the prevention study and in the regression study, heart rates were not significantly affected either by aliskiren or by losartan treatment (data not shown).

Cardiac Hypertrophy

Prevention protocol. Severe cardiac hypertrophy (Fig. 1C) developed in young untreated TGR. Aliskiren and losartan treatment significantly reduced cardiac hypertrophy in TGR, and this effect persisted after the withdrawal of aliskiren treatment, while cessation of the treatment with losartan re-

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sulted in a significant increase in cardiac hypertrophy compared with aliskiren.

Regression protocol. Strong cardiac hypertrophy was present in adult untreated TGR, which significantly exceeded that of all other groups and was similarly reduced with aliskiren and losartan treatment (Fig. 1D). While discontinuation of losartan treatment again caused a significant increase in cardiac hypertrophy, cardiac hypertrophy remained suppressed in aliskiren-treated rats.

Proteinuria

Prevention protocol. Because proteinuria and albuminuria are known to develop later in life in TGR, they were not evaluated in young TGR (prevention protocol).

Regression protocol. At week 1 (3 mo of age), proteinuria in TGR groups was significantly higher than in HanSD (Fig. 2A). Both aliskiren and losartan similarly depressed protein excretion at week 5 (Fig. 2B). Interestingly, at week 10 the effect of losartan on proteinuria was significantly weaker than that of aliskiren (Fig. 2C). At the end of the washout period (week 12), proteinuria in losartan-treated TGR did not differ from untreated TGR, while it remained decreased in aliskiren-treated TGR (Fig. 2D). Similar results were obtained with regard to albuminuria (data not shown).

Angiotensin II Levels

Prevention protocol. In the groups of both HanSD and untreated TGR, no significant differences in ANG II levels between values “before” and “after” the washout period were found; therefore the results from these two periods were pooled. Left ventricular ANG II concentrations were not significantly different among all groups examined (data not shown). As depicted in Fig. 3A, kidney ANG II levels in untreated and losartan-treated TGR significantly exceeded those of HanSD. Aliskiren treatment significantly suppressed kidney ANG II levels to the levels found in control HanSD.

Fig. 1. A and B: mean arterial pressure (MAP) in the prevention protocol (A) and in the regression protocol (B) in the course of the experiment in heterozygous male Ren-2 transgenic rats (TGR). C and D: cardiac hypertrophy (left ventricle weight/tibia length) in the prevention protocol (C) and in the regression protocol (D). *P < 0.05 vs. Hannover Sprague-Dawley rats (HanSD); #P < 0.05 vs. untreated TGR; @P < 0.05 vs. corresponding treated TGR group.
Similar to kidney ANG II, plasma ANG II levels in untreated TGR significantly exceeded those of HanSD (Fig. 3C). Whereas plasma ANG II levels were normalized with aliskiren treatment to control levels in HanSD, losartan increased plasma ANG II levels far above those of untreated TGR. These increased levels after losartan treatment are consistent with the absence of the short-loop negative feedback effect of AT1 receptor activation on renin secretion and circulating ANG II levels (33), while lower renal levels most probably result from displacement of ANG II from AT1 receptor, which is the prevailing receptor in the kidney. After the washout period, plasma ANG II in losartan-treated TGR fell progressively but remained significantly higher than that in the aliskiren-treated group.

Histology

Prevention protocol. Morphological changes in renal parenchyma were mild. Glomerular involvement ranged from slight focal segmental mesangial expansion to adhesion formation; sporadically small segmental sclerotization of glomerular tuft was found in the untreated TGR group (Fig. 4). Aliskiren was as effective as losartan in reducing GSI (Fig. 5A). After the washout period, there was a slight increase in GSI with both treatments, which did not reach statistical significance. Treatment with aliskiren did not significantly improve GSI in HanSD.
On the ultrastructural level the foot processes of podocytes in untreated TGR appeared wider than in control animals and revealed patchy fusion. Treatment with aliskiren and losartan restored normal podocyte architecture, with no significant differences between the two treatment regimens. Morphometric analysis also did not show significant differences between aliskiren- and losartan-treated animals. After the washout period a significant increase of consecutive cross-sectional glomerular area in losartan-treated rats versus aliskiren-treated TGR was found ($P < 0.05$) (Fig. 5C). Regression protocol. Morphological changes in renal parenchyma were more pronounced than in the prevention study and corresponded to focal segmental glomerulosclerosis. There was a twofold increase of GSI in untreated TGR compared with control HanSD (Fig. 5B). Both aliskiren and losartan decreased GSI to the values of control animals, with no significant changes after the washout period. Ultrastructurally, more advanced degenerative changes of podocytes were found in untreated TGR. Larger fusion of podocyte foot processes, their microvillous transformation, and electron-dense granules within the cytoplasm were found in some animals. Aliskiren and losartan similarly restored podocyte morphology (Fig. 4, C and D), the effect being the same after the washout period. Morphometric analysis revealed significant increase in consecutive glomerular area in the untreated TGR group, while aliskiren or losartan similarly decreased this parameter to the values found in HanSD (Fig. 5D). While the glomerular area was still normalized after the washout period in aliskiren-treated TGR, there was a significant increase in glomerular area of losartan-treated TGR compared with aliskiren-treated TGR. A strong correlation between glomerular size ($x$) and proteinuria ($y$) in rats from all four groups has been found ($r = 0.916$, $y = 0.015x - 118.9$; $P < 0.01$).

Fig. 3. Plasma and kidney angiotensin II in the prevention protocol (A, C) and in the regression protocol (B, D) at the end of the experiment in heterozygous male Ren-2 TGR. *$P < 0.05$ vs. control HanSD; #*$P < 0.05$ vs. untreated TGR; @* $P < 0.05$ vs. corresponding treated TGR group; §$P < 0.05$ vs. all other groups.
DISCUSSION

There are several important findings in this study. Aliskiren not only prevented the rise of BP in young TGR in the prehypertensive state (prevention study) but also reduced BP to normotensive values in adult TGR with established hypertension (regression study). These BP-lowering effects persisted for at least 12 days after treatment withdrawal in both young and adult TGR. Moreover, in adult TGR aliskiren was superior to losartan in reducing proteinuria (this effect being strongly correlated with glomerular morphology, evaluated as the size of glomerular area), while in young TGR aliskiren was also able to suppress plasma and tissue ANG II levels in a long-lasting manner.

It is not surprising that in most of the evaluated parameters, the results of the two treatments are very similar, since both treatments affect the action of ANG II—although at two different steps in the RAS cascade.

Since there have been no data comparing BP effects of aliskiren and losartan in Ren-2 TGR, the dose of losartan that would provide a BP-lowering effect comparable to aliskiren was used. Since our preliminary experiments (unpublished results) showed that the generally accepted dose of losartan (10 mg·kg⁻¹·day⁻¹) decreased BP in Ren-2 rats under HanSD levels, we used half the dose (5 mg·kg⁻¹·day⁻¹) in the present study.

Our data show that blocking the RAS with the direct renin inhibitor aliskiren at its rate-limiting step is as effective as the blockade of this system at its terminal step. In contrast, Whaley-Connell et al. (36, 38) reported that 3-wk treatment with irbesartan (30 mg·kg⁻¹·day⁻¹·ip) in 6- to 9-wk-old Ren-2 rats caused a greater reduction in BP than aliskiren treatment (50 mg·kg⁻¹·day⁻¹·ip). However, using telemetry for BP measurement, Feldman et al. (7) found a substantial BP reduction with aliskiren treatment (10 mg·kg⁻¹·day⁻¹·ip) that was comparable to our results. The reason for these discrepant results might be related to the tail cuff method of BP measurement used in the former experiment. Higher efficacy of irbesartan over losartan could be explained by their different bioavailability after oral or intraperitoneal administration and the degree of metabolism (3); moreover, one cannot exclude the different ways of application of both drugs.

In the prevention protocol, the BP-lowering effect of aliskiren persisted for at least 12 days after withdrawal of treatment, while after losartan withdrawal BP rose rapidly to a level not significantly different from that of untreated TGR. Similar results were obtained in the regression protocol, i.e., increase of BP in losartan-treated animals, but after the cessation of losartan treatment the BP increase was much slower compared with the prevention protocol and, finally, BP did not reach the levels of untreated TGR. In hypertensive patients, Oh et al. (24) found that BP reduction was maintained for 2 wk after withdrawal of aliskiren treatment and coincided with the persistent reduction of plasma renin activity. In our study, the BP-lowering effects paralleled the suppression of cardiac hy-
pertrophy and in young animals also reductions of plasma and renal ANG II concentrations. These levels were significantly higher in young than in adult untreated TGR, which is in good agreement with our previous data (11) showing that both plasma and renal ANG II concentrations decrease with age in conscious TGR. However, Lee et al. (20) reported unchanged plasma ANG II and Kopkan et al. (15) reported even decreased plasma ANG II concentrations in anesthetized Ren-2 animals. These discrepant values might be due to the different status of animals, our results being obtained from conscious (decapitated) animals while the others used anesthetized animals. Aliskiren has been shown to distribute extensively to the kidneys (7). In addition, Feldman et al. (8) have shown that, in contrast with kidney, aliskiren level in plasma was not detectable. Moreover, there is evidence that aliskiren accumulates in renin secretory granules, and after exposure of renin-secreting cells to aliskiren aliskiren-bound renin is released (17). Furthermore, Batenburg et al. (1) reported that the half-life of (pro)renin is increased after aliskiren binding in rat aortic vascular smooth muscle cells. These facts might explain why the BP-lowering effect of aliskiren persists for a long time after withdrawal of treatment.

As can be seen from our results in both prevention and regression protocols, cardioprotection is BP dependent in Ren-2 rats; the same effect on cardiac hypertrophy was achieved with both treatments. However, during the washout period cardiac weight remained stable in aliskiren-treated animals with normalized BP, while after withdrawal of losartan treatment BP rose quickly and was followed by an increase in cardiac hypertrophy. This is in accordance with the predomi-

![Graphical representation of Glomerulosclerosis index and Glomerular area](image-url)
nating opinion that it is important to achieve normotension for slowing the progression of target organ damage.

As has been previously reported (7, 25), TGR evolve proteinuria and albuminuria at the age of 8–10 wk; therefore no evaluation of this parameter was included in our prevention protocol. Renoprotective effect of aliskiren has repeatedly been confirmed not only in humans (28) but also in a model of diabetic nephropathy in Ren-2 animals (7) and in double transgenic rats (5, 29). In the present study, the antiproteinuric effects of aliskiren and losartan were similar after 5 wk of treatment, while after 10 wk of treatment aliskiren showed stronger antialbuminuric (data not shown) and antiproteinuric effects than losartan. Although glomerulosclerosis, as a marker of renal damage, was the same at the end of the experiment, there was substantial increase in glomerular area of losartan-treated TGR, which strongly correlated with proteinuria. In contrast, Whaley-Connell et al. (38) reported similar effects of irbesartan and aliskiren on podocyte injury in young Ren-2 rats, while irbesartan had a greater BP-lowering effect. Our findings are in agreement with the findings of Ijpellar et al. (13), who found that glomerular hypertrophy precedes albuminuria and proteinuria in Munich-Wistar-Frömter (MWF) rats. It appears that the expansion of glomerular tuft requires adaptation of podocytes to cover a larger area of glomerular capillary wall (16), and this situation requires reorganization of the podocyte cytoskeleton. Increased workload then leads to podocyte damage. Moreover, it is known that ultrastructural changes appear at least 3 wk after the immunologic changes (23, 35), and our results on podocyte injury in Ren-2 TGR (25) have shown that ultrastructural changes of podocytes precede morphological changes seen by light microscope.

At the end of the prevention protocol, aliskiren decreased plasma and kidney ANG II levels to those seen in control HanSD, suggesting that the depressed levels of intrarenal ANG II could be responsible for the attenuation of renal damage in aliskiren-treated TGR. However, this finding was not confirmed in the regression protocol with adult animals, although similar results were obtained in our previous study (34). Also, different data were obtained in losartan-treated animals between the prevention and regression studies. These different effects could be explained by the different actions of both drugs in the induction and maintenance phase of hypertension, since it is known that interventions applied in young animals are usually more effective than those applied later in life (39). The beneficial effect of aliskiren over losartan might be related to the fact that aliskiren decreases (pro)renin receptor gene expression, as has been shown by Feldman et al. (7). Whaley-Connell et al. (38) found similarly decreased ANG II content with both aliskiren and irbesartan, while aliskiren significantly reduced AT1 receptor levels compared with irbesartan. Therefore, the effect of aliskiren on (pro)renin receptor (9, 12) and AT1 receptor levels rather than tissue ANG II levels could be responsible for the beneficial effects of aliskiren. Further studies will be necessary to clarify these questions.

In conclusion, although the renin inhibitor aliskiren in many aspects exerts effects similar to those of the angiotensin receptor blocker losartan, it has several beneficial properties that may make it superior to classical AT1 receptor blockers in clinical practice, including its long-term effects on BP, cardiac hypertrophy, and proteinuria accompanied by normalized glomerular area.

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DISCLOSURES

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REFERENCES

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