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Prof. Dr. med. Rolf A. K. Stahl

AT1-Antagonismus im Vergleich zur Renininhibition bei chronischer Niereninsuffizienz der Maus: Angiotensin II ist der entscheidende Progressionsfaktor

Dissertation

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Christoph Fraune
aus Kassel

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Prüfungsausschuss, zweite/r Gutachter/in: Prof. Dr. H. Ehmke

~~**Prüfungsausschuss, dritte/r Gutachter/in:**~~

Inhaltsverzeichnis

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I. Publikationen

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AT_1 antagonism and renin inhibition in mice: pivotal role of targeting angiotensin II in chronic kidney disease

Christoph Fraune,[1] Sascha Lange,[1] Christian Krebs,[1] Alexandra Hölzel,[1] Jana Baucke,[1] Nevena Divac,[2] Edzard Schwedhelm,[3] Thomas Streichert,[4] Joachim Velden,[5] Ingrid M. Garrelds,[6] A. H. Jan Danser,[6] Anne-Roos Frenay,[7] Harry van Goor,[7] Vera Jankowski,[8] Rolf Stahl,[1] Genevieve Nguyen,[9] and Ulrich Otto Wenzel[1]

[1]*Division of Nephrology, Department of Medicine, University Hospital Hamburg-Eppendorf, Hamburg, Germany;* [2]*Department of Pharmacology, School of Medicine, University of Belgrade, Belgrade, Serbia;* [3]*Department of Clinical Pharmacology, University Hospital Hamburg-Eppendorf, Hamburg, Germany;* [4]*Department of Laboratory Medicine, University Hospital Hamburg-Eppendorf, Hamburg, Germany;* [5]*Nierenregister am Institut für Pathologie, University Hospital Hamburg-Eppendorf, Hamburg, Germany;* [6]*Division of Vascular Medicine and Pharmacology, Department of Internal Medicine, Erasmus MC, Rotterdam, The Netherlands;* [7]*Department of Pathology and Medical Biology, University Medical Centre Groningen, University of Groningen, Groningen, The Netherlands;* [8]*Division of Nephrology, Charité-Universitätsmedizin Berlin, Campus Benjamin Franklin, Medizinische Klinik IV, Berlin, Germany; and* [9]*College de France, Paris, France*

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Fraune C, Lange S, Krebs C, Hölzel A, Baucke J, Divac N, Schwedhelm E, Streichert T, Velden J, Garrelds IM, Danser AH, Frenay A, van Goor H, Jankowski V, Stahl R, Nguyen G, Wenzel UO. AT_1 antagonism and renin inhibition in mice: pivotal role of targeting angiotensin II in chronic kidney disease. *Am J Physiol Renal Physiol* 303: F1037–F1048, 2012. First published July 11, 2012; doi:10.1152/ajprenal.00672.2011.—The role of the renin-angiotensin system in chronic kidney disease involves multiple peptides and receptors. Exerting antipodal pathophysiological mechanisms, renin inhibition and AT_1 antagonism ameliorate renal damage. However, it is unclear which mechanism exerts better nephroprotection. We compared the renin inhibitor aliskiren with the AT_1 antagonist losartan in mice with chronic kidney disease due to renal ablation. Doses were adjusted to equipotent inhibition of the renin-angiotensin system, determined via a dose-response quantifying plasma and renal renin expression. Six-week treatment with either 500 mg/l drinking water losartan or 50 $mg \cdot kg^{-1} \cdot day^{-1}$ aliskiren significantly decreased albuminuria, glomerular damage, and transcription rates of renal injury markers to a similar extent. An array analysis comparing renal gene expression of losartan- and aliskiren-treated mice evaluating >34,000 transcripts demonstrated regulation for 14 genes only, with small differences. No superior nephroprotection was found by combining losartan and aliskiren. Compared with plasma concentrations, aliskiren accumulated ~7- to 29-fold in the heart, liver, lung, and spleen and ~156-fold in the kidney. After withdrawal, plasma concentrations dropped to zero within 24 h, whereas renal tissue concentrations declined slowly over days. Withdrawal of aliskiren in mice with chronic kidney disease revealed a significantly delayed re-increase in albuminuria compared with withdrawal of losartan. This study demonstrates equieffective nephroprotection of renin inhibition and AT_1 antagonism in mice with chronic kidney disease without additional benefit of combination therapy. These observations underscore the pivotal role of targeting ANG II to reduce renal injury.

5/6 nephrectomy; renal impairment

THERE IS EVOLVING COMPLEXITY in the renin-angiotensin system (RAS) because of the identification of a spectrum of bioactive angiotensin peptides and receptors that interact with these fragments. ANG II has long been considered the major bioactive contributor to chronic kidney disease (CKD). Chronic ANG II infusion induces albuminuria and renal injury (23). Conversely, angiotensin-converting enzyme (ACE) inhibition and ANG II type 1 receptor (AT_1) antagonism are nephroprotective (1, 4, 29). However, it is controversial whether this efficacy is due to inhibition of ANG II production, blockade of the AT_1 receptor, a shift of ANG II toward AT_2 receptor transduction, or the continued or even increased presence of other bioactive angiotensin peptides that interact with receptors beyond the AT_1 and AT_2 receptor.

Renin inhibition suppresses the production of all angiotensin peptides (27), providing a unique approach to define the role of angiotensin peptides in the progression of CKD (20). Due to the high species specificity of the renin-angiotensinogen reaction, animal data facilitating a more detailed insight into the nephroprotective effects of renin inhibition are scarce. In double transgenic rats (dTGR) expressing both the human renin and angiotensinogen gene (11), renin inhibition decreases blood pressure and albuminuria (33). However, given the exceptionally high ANG II levels in these animals (up to 20 times normal) causing death within 7 wk without treatment (33), as well as the absence of the RAS feedback mechanisms, they are ill-suited for studying normal physiological events.

Renin inhibition and AT_1 antagonism have been shown to improve albuminuria and end-organ damage. Several reports suggest that the beneficial effects of aliskiren are due to a decrease in ANG II production, while others have suggested an additive effect of aliskiren to antagonize the increased plasma renin activity (PRA) due to AT_1 blockade (9). Renin inhibition has been suggested to be superior to ACE inhibition or AT_1 antagonism in previous studies (22). Moreover, the combination of AT_1 antagonism and renin inhibition has shown superior protection against renal fibrosis in mice (8, 39–40). However, in none of these studies was RAS inhibition quantified as in the present study, nor did the monotherapy exceed low-dose treatment. Therefore, we performed for the first time extensive dose-response curves for aliskiren and losartan and asked three questions. *1*) What is the nephroprotective efficacy of aliskiren compared with equipotent high-dose treatment with the AT_1

Address for reprint requests and other correspondence: U. Wenzel, Div. of Nephrology, Dept. of Medicine, Universitätsklinikum Hamburg-Eppendorf, Martinistraβe 52, 20246 Hamburg, Germany (e-mail: wenzel@uke.de).

antagonist losartan? *2*) Does aliskiren on top of losartan exert more nephroprotection than the monotherapies by i.e., inhibiting the increased PRA in response to losartan? *3*) Are there differences between aliskiren and losartan after withdrawal of treatment? Renal ablation, a mainstay in experimental kidney research, was used for CKD induction in FVB/N mice. This model features all clinical hallmarks of CKD, including substantial albuminuria, uremia, and glomerulosclerosis (3). Furthermore, mice serve as the ideal target to study renin inhibition, because the IC_{50} of aliskiren for mouse renin (4.5 nM) is considerably closer to human renin (0.6 nM) than is rat renin (80 nM) (10, 27, 38).

Emerging evidence indicates sustained effects of aliskiren beyond withdrawal (31, 35). Besides its long plasma half-life in humans, speculations about any putative renal accumulation of aliskiren were held to explain this phenomenon (10). We compared plasma concentrations with various tissue levels of aliskiren and analyzed its effect compared with losartan on albuminuria after withdrawal.

Using renal ablation in FVB/N mice as a model for CKD, this is the first study comparing renin inhibition and AT_1 antagonism based on equipotent RAS inhibition in nontransgenic animals. This facilitates a meaningful evaluation of the nephroprotective efficacy of renin inhibition during treatment as well as of prolonged effects after its withdrawal.

MATERIALS AND METHODS

All experiments started in 10-wk-old male FVB/N mice. Aliskiren (Novartis) was administered via osmotic minipump infusion (model 2002, Alzet Osmotic Pumps; DURECT), and losartan (Sandoz) was dissolved in the drinking water. All animal procedures were approved by the local animal committee and were in accord with national and institutional animal care guidelines.

Dose-response curve and effect on angiotensin fragments. To identify dosages for losartan and aliskiren yielding equipotent RAS inhibition, healthy FVB/N mice were treated with increasing dosages of both drugs for 14 days before renal renin was quantified with real-time PCR for RNA and immunostaining for protein levels. Plasma renin concentration (PRC) was measured as described elsewhere (25). PRC was determined by measuring ANG I formation in the presence of excess angiotensinogen after diluting of the samples to get rid of the aliskiren. Plasma angiotensin peptides were analyzed by matrix-assisted laser desorption/ionization (MALDI) time-of-flight/time-of-flight (TOF/TOF) high-resolution tandem mass spectrometry as previously described (19). The detection limit was 4×10^{-16} mol. In addition, plasma ANG II levels were also quantified with a conventional enzymatic immunoassay by an ANG II enzyme immunoassay kit from SpiBio (Massy, France). ANG II from the plasma was concentrated by using phenyl cartridges (SpiBio) and water and was eluated by methanol. The eluate was lyophilized by vacuum centrifugation at 4°C. The kit was used according to the manufacturer's protocol. For blood collection, an inhibitor cocktail (Protease Inhibitor Cocktail, Sigma) was used to prevent generation and/or degradation of ANG II.

Comparison of aliskiren and losartan in CKD. Two weeks before the start of the experiment, two-thirds of the left kidney was removed as described (3). At *day 0*, renal ablation was completed by contralateral uninephrectomy. Four weeks thereafter, albuminuria and blood urea nitrogen (BUN) were measured and mice were randomized into three groups: untreated, losartan, and aliskiren. In the subsequent 6 wk, 500 mg/l drinking water losartan or 50 $mg \cdot kg^{-1} \cdot day^{-1}$ aliskiren were administered. The losartan dose for a 30-g mouse drinking 10 ml/day averages ~167 $mg \cdot kg^{-1} \cdot day^{-1}$. Mice were euthanized after *week 10* to collect organs and blood for further analyses. In a second set of experiments, double blockade with losartan plus aliskiren was performed concomitantly to both monotherapies.

Half-life of aliskiren in the kidney, heart, and plasma. To determine the tissue and plasma half-life of aliskiren, healthy FVB/N mice were treated with 50 $mg \cdot kg^{-1} \cdot day^{-1}$ aliskiren for 14 days. Aliskiren was withdrawn, and five mice each were euthanized immediately (*day 0*) or 1, 3, or 7 days thereafter.

Plasma levels of ANG II after withdrawal of losartan and aliskiren. In another set of experiments, healthy FVB/N mice were treated with aliskiren and losartan for 14 days. Aliskiren and losartan were withdrawn, and five mice each were euthanized immediately (*day 0*) or 1 or 3 days thereafter. ANG II plasma levels were measured by ELISA.

Albuminuria. Mice were placed into metabolic cages for a 6-h urine collection. Urine albumin was measured using a commercially available mouse-specific ELISA (E90–134; Bethyl Laboratories) and urine creatinine by an autoanalyzer (Hitachi 717; Roche). Albuminuria was calculated as milligrams albumin per milligram creatinine.

Plasma values. Before treatment, mice were slightly anesthetized with carbon dioxide and the retroorbital sinus was punctured with a glass capillary tube for heparinized blood collection. At death, blood was drawn intracardially. BUN and plasma cholesterol were determined by an autoanalyzer (Hitachi 717).

Blood pressure. Systolic blood pressure was measured in conscious mice using computerized tail-cuff plethysmography (Process Control Blood Pressure 2900 series; TSE Systems) as described elsewhere (23). Mice were trained to get used to this procedure in advance, and initial measurements were not incorporated.

Histopathological analysis. Kidney tissue was fixed with 4% neutral buffered formaldehyde, embedded in paraffin, and sectioned. Sections were stained for light microscopy with periodic acid-Schiff reagent. Glomerular injury was evaluated using a semiquantitative scale between 0 and 3. Proteinaceous casts were counted in the whole cortex. For assessment of interstitial injury, nonoverlapping cortical areas were analyzed by overlaying a grid containing 40 points. For renin immunohistochemistry, a polyclonal antibody was used as described elsewhere (24). Juxtaglomerular renin was quantified using a semiquantitative scale from 0 to 3. The size of the juxtaglomerular apparatus was found by computer-aided assessment.

Real-time PCR analyses. Total RNA of the renal cortex was prepared according to standard laboratory methods. Quantitative real-time PCR analysis (StepOnePlus, Applied Biosystems) was performed using SYBR Green dye (Qiagen).

The following primers were used: 18S: forward 5′-CAC GGC CGG TAC AGT GAA AC-3′, reverse 5′-AGA GGA GCG AGC GAC CAA A-3′; fibronectin: forward 5′-AGA CCA TAC CTG CCG AAT GTA G-3′, reverse 5′-GAG AGC TTC CTG TCC TGT AGA G-3′; plasminogen activator inhibitor (PAI)-1: forward 5′-GGA CAC CCT CAG CAT GTT CA-3′, reverse 5′-TCT GAT GAG TTC AGC ATC CAA GAT-3′; renin: forward 5′-GCT CTG GAG TCC TTG CAC CTT-3′, reverse 5′-CTT GAG CGG GAT TCG TTC AA-3′; and renin receptor: forward 5′-TCA TTC GAC ACA TCC CTT GTG-3′, reverse 5′-AGG TTA TAG GGA CTT TGG GTG TTC T-3′.

Determination of aliskiren in plasma and tissue. Aliskiren in plasma (25 μl) and tissue (2–10 mg wet wt) samples was determined using compound 21 as an internal standard (IS) by liquid chromatography-tandem mass spectrometry (LC-MS/MS) according to Wan et al. (37). Analytes were chromatographed on a Synergy Polar RP column [30 × 2-mm ID, Phenomenex, Torrance, CA, at a flow rate of 0.4 ml/min; 0- 0:30 min 10% B, 0:30–3 min increase to 90% B, 3–3:30 min 90% B, reequilibration at 10% B until 8 min, 10 mM ammonium acetate (A), acetonitril (B)]. The following transitions were used for the quantitative determination of analytes (ESI+): *m/z* 552.4 -> 418.4 and *m/z* 552.4 -> 436.4 for aliskiren, and *m/z* 476.4 -> 293.2 and *m/z* 476.4 -> 402.2 for IS.

Microarray analysis. Renal tissue from renal-ablated mice after 6-wk treatment with either 500 mg/l losartan or 50 $mg \cdot kg^{-1} \cdot day^{-1}$ aliskiren (n = 5 each) were used for the experiments. Affymetrix

GeneChip Mouse Genome 430 2.0 Arrays (Affymetrix) containing ~34,000 genes and expressed sequence tags (ESTs) were used. Target preparation and hybridization with Gene Chip microarrays were performed as described by the manufacturer. Microarrays were scanned with Affymetrix GeneChip Scanner 7G. Expression signals were RMA background corrected and quantile normalized using Affymetrix Expression Console Software (version 1). Significance of gene regulation was determined by application of a Welch's unpaired *t*-test (R statistical platform, ver. 2.11.0), followed by a sample permutation correction for multiple testing. Genes were declared as significant when exhibiting a permutation-derived P value <0.05 and an absolute signal-log-ratio >0.8 (fold-change of 1.75). The data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Seris accession number GSE 34552. (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE34552).

Statistical analysis. Data are expressed as the median [25th percentile; 75th percentile], unless otherwise indicated. In the case of an uneven distribution, laboratory parameters were logarithmically transformed for statistical analyses. Univariate ANOVA was performed using SPSS Statistics 18.0 (SPSS). All intergroup comparisons were performed pairwise using a least significant difference posttest. For the comparison between study groups having received renal ablation, the albuminuria before the start of therapy was used as covariate (ANCOVA). For the experiment of withdrawal of aliskiren and losartan in CKD, albuminuria before the start of therapy and at the day of withdrawal of therapy were used as covariates. Significance was considered for an error of $\alpha < 5\%$ ($P < 0.05$).

RESULTS

Dose-response curve. Interfering with the RAS stimulates renal renin expression due to inhibition of the ANG II-mediated negative-feedback loop. Thus quantifying renin allows one to estimate the magnitude of the RAS blockade (27). Equieffective RAS inhibition with losartan and aliskiren was identified via a dose-response measuring the reactive renin rise in healthy control mice. Renin RNA levels, plasma renin concentration, and renin immunohistochemistry revealed a dose-dependent increase for both drugs (Fig. 1*A*). Plasma concentrations and RNA levels of renin correlated well. Equipotent RAS inhibition was approximately observed for 500 mg/l losartan and 50 mg·kg^{-1}·day^{-1}

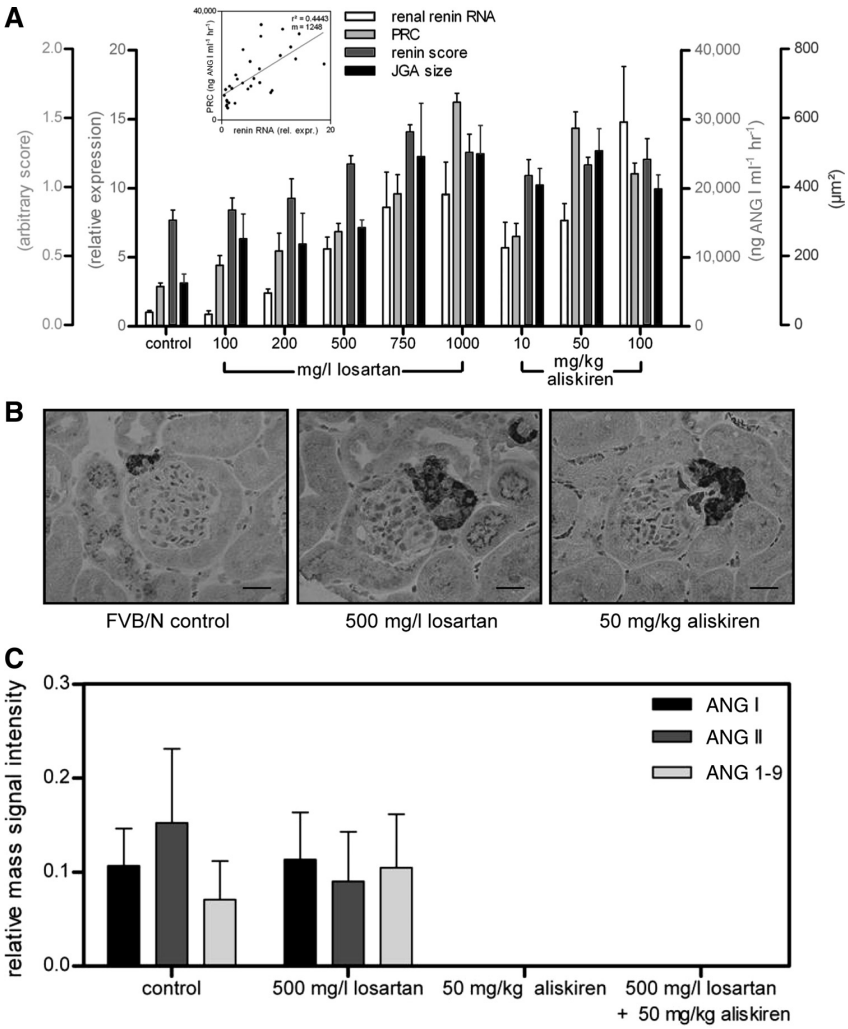

Fig. 1. To adjust losartan and aliskiren to equipotent inhibition of the renin-angiotensin system (RAS), renin was quantified by RNA levels, plasma renin concentration, and immunohistochemistry quantifying the renin-positive juxtaglomerular area (JGA) both morphometrically and with an arbitrary score (*A*; n = 2–16/group). Both drugs demonstrated an increase in renin in a dose-dependent manner. Plasma renin concentration (PRC) correlated well with renin RNA levels. Losartan (500 mg/l) and 50 mg·kg^{-1}·day^{-1} aliskiren increased renin RNA as well as protein expression comparably and were thus considered to inhibit the RAS equipotently. Representative 400-fold magnifications of renin immunohistochemistry of JGA of healthy FVB/N control mice and mice treated with losartan or aliskiren are shown (*B*). Plasma angiotensin fragments of controls, losartan-, and aliskiren-treated mice (n = 4–6/group) are shown (*C*). Values are means ± SE.

aliskiren. In this regard, the presumable overestimation of renin protein measurements in the immunoassay, promoted by conformational changes in prorenin during aliskiren therapy and the technical challenge to measure PRC in mice, was taken into account (6). Figure 1*B* depicts representative renin immunohistochemistry. Furthermore, renin inhibition decreased plasma concentrations of angiotensin fragments sufficiently (Fig. 1*C*). ANG I, II, and 1–9 were undetectable after aliskiren, but unaltered after losartan treatment.

Similar nephroprotective properties of aliskiren and losartan. Equipotent losartan and aliskiren treatment in mice with CKD was begun 4 wk succeeding renal ablation. Before therapy, no difference was found in the removed kidney weight, BUN, and albuminuria between all groups, indicating similar induction of CKD (Table 1). Throughout the subsequent 6 wk, albuminuria increased to 16.22 [9.86; 67.40] in untreated renal-ablated mice and progressively decreased to 0.95 [0.45; 2.64] in losartan- and 0.59 [0.42; 1.47] in aliskiren-treated mice (Fig. 2*A*). This highly significant reduction was of comparable magnitude in both treatments (Fig. 2*B*). Intratubular proteinaceous casts, the morphological correlate of proteinuria, were observed in renal-ablated mice (Fig. 2*D*). Quantification revealed a significant reduction of casts for both treatments (Fig. 2*C*). Elevated plasma cholesterol levels indicated the nephrotic range of albuminuria (Fig. 2*E*). Both treatments reduced cholesterol levels significantly.

Glomerular and interstitial damage. The abundant glomerular damage in untreated renal-ablated mice was significantly attenuated by losartan and aliskiren to a comparable extent (Fig. 3*A*). The interstitial area (Fig. 3*B*) was increased after renal ablation and not reduced by any therapy. Figure 3*C* depicts representative micrographs.

RNA expression. Renal ablation downregulated renin RNA (0.16 [0.10; 0.33]) compared with controls (Fig. 4A). Treatment with losartan (1.36 [0.96; 2.45]) or aliskiren (1.35 [0.54; 1.57]) leads to an approximately eightfold increase in renin compared with untreated renal-ablated mice, confirming equipotent RAS inhibition in CKD mice for the doses evaluated before in healthy mice. The RNA expression of the (pro)renin receptor was unaltered (Fig. 4*B*). Both treatments significantly decreased profibrotic markers such as PAI-1 (Fig. 4*C*) and fibronectin (Fig. 4*D*).

Renal function and systolic blood pressure. Mortality did not differ between untreated and losartan- or aliskiren-treated mice (Table 2). Renal ablation decreased renal function, illustrated by increased BUN levels. Both drugs had no significant effect on BUN levels. Systolic blood pressure increased moderately in renal-ablated mice. This was attenuated by both treatments. We cannot differentiate from our data whether the observed nephroprotection is due to the small decrease in blood pressure or blood pressure-independent effects of renin inhibition and AT_1 receptor blockade since we did not include a non-RAS inhibitor treatment group. However, there is overwhelming evidence from experimental and clinical data that, especially in CKD with proteinuria and the renal ablation model, RAS inhibition is superior to non-RAS inhibition. Analogous to blood pressure, the relative heart weight was increased in untreated, but reduced in treated renal-ablated mice.

Microarray analysis of renal gene expression. Microarray analysis of renal gene expression comparing 6-wk losartan or aliskiren treatment in mice with CKD revealed significant regulation with a signal log ratio (SLR) >0.8 of 14 genes only with a maximum of a 2.58-fold change (Fig. 5). No major pathway differentially expressed by renin inhibition or AT_1 antagonism can be identified since the genes are a mixture of matrix, transporter cytokine, receptor, and transduction proteins as well as unidentified genes. In contrast, significant regulation was found of 157 genes for aliskiren and 127 genes for losartan treatment both compared with untreated mice (data not shown).

Double blockade. Due to the high variability of the renal damage caused by renal ablation, mice of the double blockade were only compared with mice treated with aliskiren or losartan monotherapy in the same set of experiments. Double blockade was not superior, as assessed for albuminuria (Fig. 6), BUN, systolic blood pressure, gene expression of renin and fibronectin, and glomerular sclerosis (Table 3). Although plasma potassium levels were slightly higher in ablated mice with and without RAS blockade, no significant difference was found between the different groups (controls 5.3 ± 0.1, ablation 6.3 ± 0.5, ablation+losartan 6.4 ± 0.4, ablation+aliskiren 6.1 ± 0.4, ablation+losartan+aliskiren 6.6 ± 0.2 mmol/l).

Plasma and tissue levels of aliskiren. In healthy FVB/N mice, 50 $mg \cdot kg^{-1} \cdot day^{-1}$ treatment yielded aliskiren plasma levels of 0.60 μg/ml [0.50; 0.71] (Fig. 7). In contrast, aliskiren tissue levels were between 4.00 and 17.50 μg/g wet weight for the heart, lung, liver, and spleen. This revealed ~7- to 29-fold higher levels in these tissues compared with plasma. Interestingly, renal tissue levels [93.85 μg/g (67.70; 122.25)] were ~156-fold higher than plasma levels. Comparing micrograms per milliliter and per gram is reasonable since 1 gram matches roughly 1 ml (7).

Aliskiren was given to healthy FVB/N mice, the pumps were removed after 14 days, and mice (n = 5 each) were immediately euthanized or at *day 1*, *3*, or *7* thereafter. Aliskiren plasma levels almost decreased to below detection within 24 h (<0.01 μg/ml) (Fig. 8*A*), whereas tissue concentrations remained detectable for several days in the kidney (Fig. 8*B*) and heart (Fig. 8*C*). ANG II reappeared already on *day 1* after cessation of therapy (Fig. 8*D*).

Withdrawal of aliskiren and losartan in mice with CKD. Six-week therapy with losartan and aliskiren reduced albuminuria

Table 1. *Before therapy 4 wk after renal ablation*

Group	n	Removed Kidney Weight, mg	Blood Urea Nitrogen, mg/dl	Albuminuria-to-Creatinine Ratio, mg/mg
Control	17	0.0	26.0 [24.0; 28.0]	0.12 [0.07; 0.47]
Ablation	21	83.0 [78.1; 94.0]	68.0 [63.5; 88.5]†	7.95 [3.24; 39.25]†
Ablation+losartan	16	87.4 [76.7; 96.9]	72.0 [62.0; 80.0]†	7.71 [3.46; 23.57]†
Ablation+aliskiren	16	80.2 [69.8; 99.7]	63.0 [54.0; 79.3]*	8.65 [5.62; 15.84]†

Values are expressed as the median [25th percentile; 75th percentile]. *$P < 0.05$ vs. control. †$P < 0.001$ vs. control.

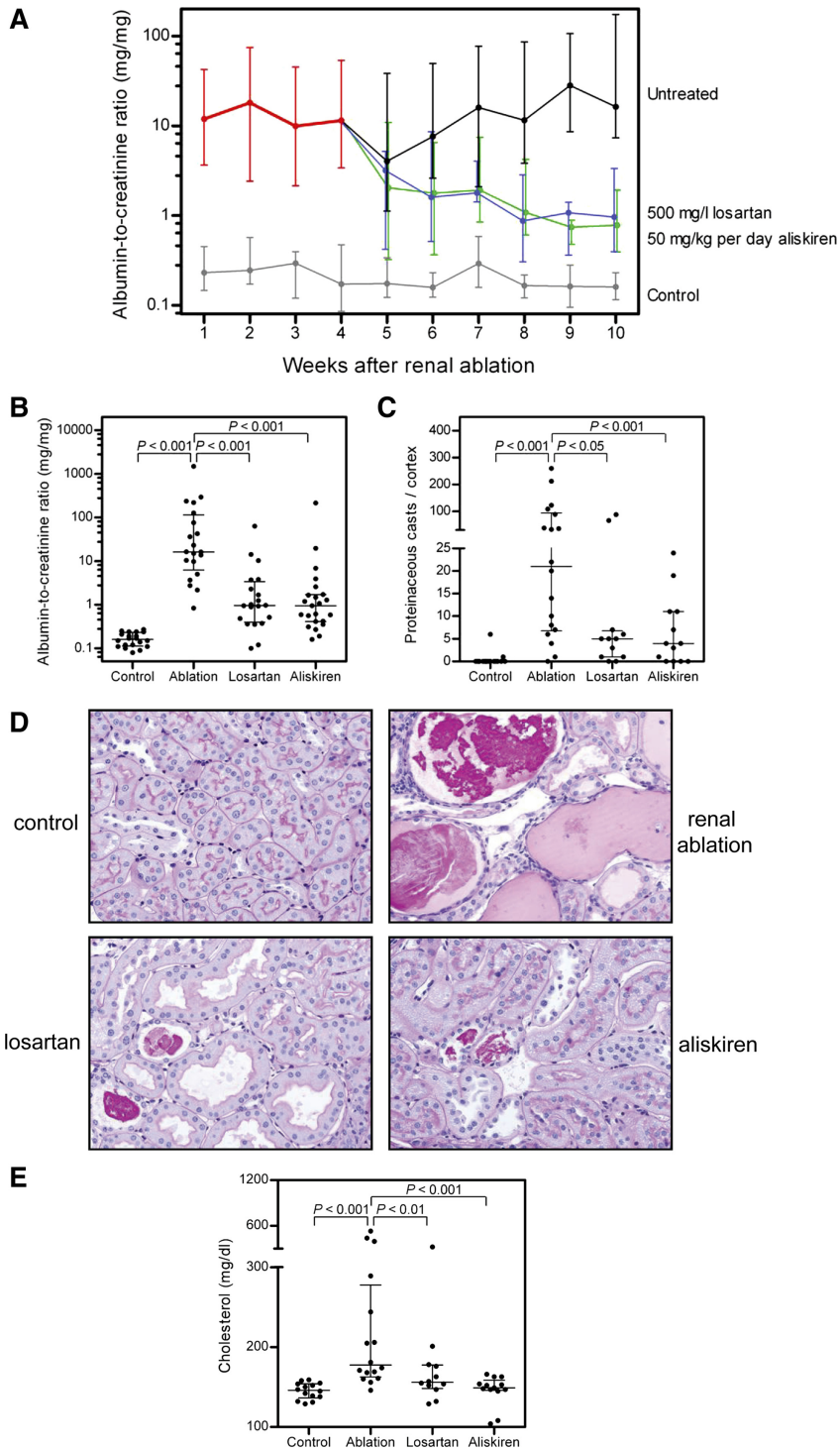

Fig. 2. Losartan and aliskiren treatment was begun 4 wk after renal ablation. Whereas albuminuria further increased in untreated mice, treatment with losartan and aliskiren progressively reduced albuminuria throughout the 6-wk treatment period (*A*). This highly significant reduction at death was of similar magnitude for both treatments (*B*). Abundant intratubular proteinaceous casts were found in untreated mice but were rare in both treatment groups (*C*). Increased plasma cholesterol in untreated mice indicated the nephrotic range of albuminuria. Both treatments reduced cholesterol levels significantly (*E*). Representative micrographs at ×400 magnification are shown (*D*). Albuminuria is shown with logarithmic scaling.

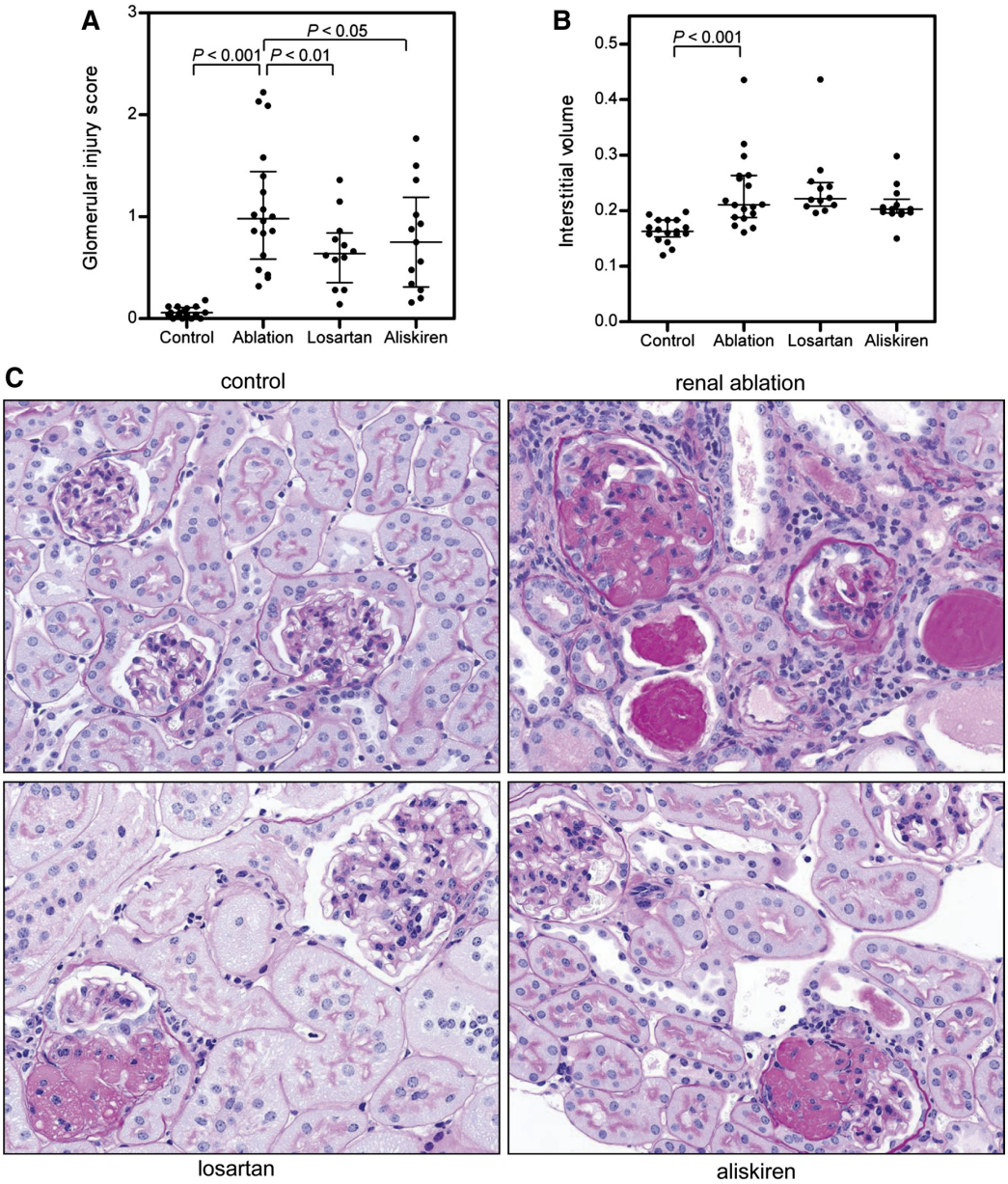

Fig. 3. Assessment of glomerular damage (*A*) and interstitial volume (*B*) was based on histopathological analysis of periodic acid-Schiff-stained kidney sections. Both parameters increased after renal ablation. Treatment with losartan or aliskiren reduced glomerular damage but had no effect on interstitial volume. Representative micrographs at ×400 magnification are shown (*C*).

in mice with CKD. Withdrawal of aliskiren and losartan revealed a re-increase in albuminuria. This re-increase was significantly lower following cessation of aliskiren than of losartan (Fig. 9*A*), suggesting the renal accumulation of aliskiren to be of pathophysiological relevance. As plasma ANG II in healthy mice reappeared already on the first day subsequent to withdrawal of aliskiren (Fig. 8*D*), the observed delayed re-increase in albuminuria in mice with CKD may not relate to plasma angiotensin levels. Since ANG II was measured by ELISA (Fig. 8*D*) and by mass spectrometry (Fig. 1), we confirm with two different methods that plasma levels of ANG II are not increased in losartan-treated mice.

DISCUSSION

The present study is the first to compare head-to-head aliskiren and losartan based on equipotent RAS blockade in mice with CKD. High-dose renin inhibition ameliorates CKD

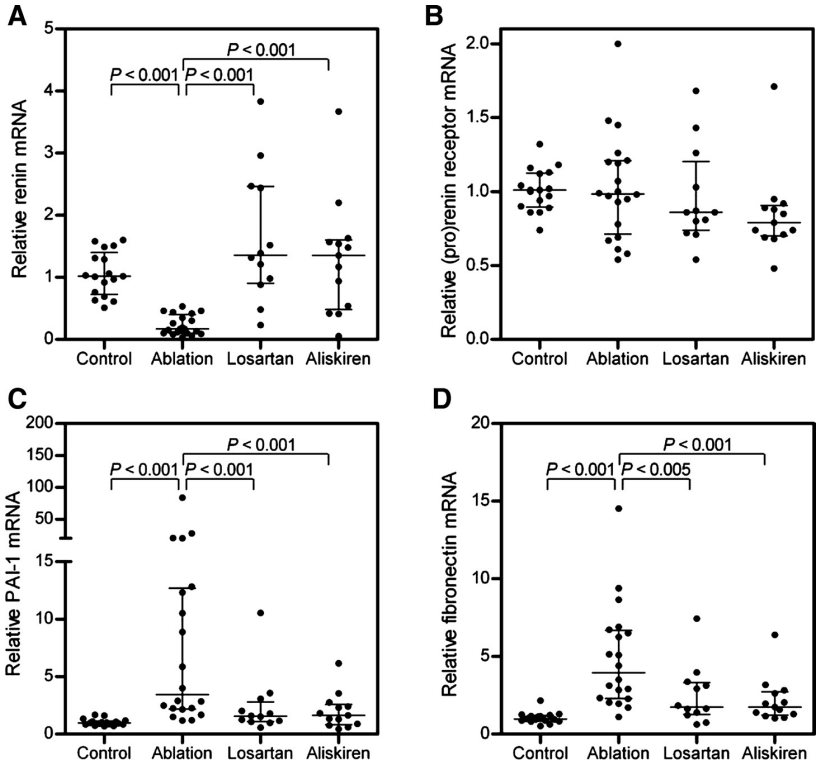

Fig. 4. Renocortical RNA levels of renin were reduced in renal-ablated mice (*A*). In comparison, treatment with losartan or aliskiren caused an ~8-fold re-increase. No changes in the RNA levels of the (pro)renin receptor were observed (*B*). The levels of plasminogen activator inhibitor 1 (PAI-1; *C*) and fibronectin (*D*) were elevated in renal-ablated mice and significantly reduced with losartan and aliskiren treatment.

to at least the same degree as does high-dose AT_1 antagonism. Administration on top of the AT_1 antagonist did not further improve CKD. We cannot confirm that other bioactive angiotensin peptides beyond ANG II play an essential role in renal injury, at least not in the renal ablation model in mice. We cannot rule out from our data that intrarenal angiotensin levels are differently regulated compared with plasma fragments. However, van Esch et al. (36) recently showed the same downregulation of renal and plasma levels of angiotensin fragments in aliskiren-treated rats. Finally, this is the first study demonstrating accumulation of aliskiren in the murine kidney and to a lower degree in the heart, lung, liver, and spleen. After withdrawal of treatment, plasma levels of aliskiren ceased within 24 h, whereas aliskiren was eliminated from both the kidney and heart slowly over several days.

Great care was taken to determine equipotent doses for aliskiren and losartan with respect to RAS inhibition to avoid any putative difference in nephroprotection to be merely attributed to incommensurable RAS blockade. Renin inhibition inhibits ANG II production whereas AT_1 antagonism blocks binding to the AT_1 receptor. Therefore, in the present study we used a downstream signal of the AT_1 receptor, which is renin expression. Binding of ANG II to the AT_1 receptor inhibits renin expression and inhibition of the RAS regardless of which position increases renin expression. As shown before, two different RAS inhibitors inhibit the RAS to the same extent if the increase in renin expression is of the same magnitude (27). Furthermore, RAS inhibition is almost maximal if a further dose increase does not increase renin expression any longer. In the present work, near-maximal doses were used to prevent additional effects of the double blockade to be simply due to underdosing of the monotherapies. In fact, in the recent AVOID trial, addition of aliskiren in patients with diabetic nephropathy pretreated with 100 mg losartan lowered proteinuria by an additional 18%, suggesting the superiority of a double blockade over monotherapy (32). However, 100 mg losartan/day is not a high-dose RAS blockade in patients. The present study, based on near-maximal doses, did not reveal superior nephroprotection by the double blockade. This clearly shows that inhibiting the increase in renin activity in response

Table 2. *At euthanasia after 6-wk therapy*

Group	Mortality after Randomization	Blood Urea Nitrogen, mg/dl	Systolic Blood Pressure, mmHg	Relative Heart Weight, mg/g
Control	0	28.5 [26.0; 35.8]	95.0 [90.0; 100.6]	4.8 [4.2; 5.2]
Ablation	3	73.5 [59.0; 94.3]†	130.0 [125.0; 140.0]†	5.4 [4.9; 5.7]†
Ablation+losartan	4	70.5 [66.0 81.3]†	108.8 [101.3; 118.8]*	4.6 [4.2; 5.2]*
Ablation+aliskiren	3	78.0 [65.0; 84.0]†	100.0 [97.5; 106.3]*	4.6 [4.4; 4.9]*

Values are expressed as the median [25th percentile; 75th percentile]. *$P < 0.01$ vs. ablation. †$P < 0.005$ vs. control.

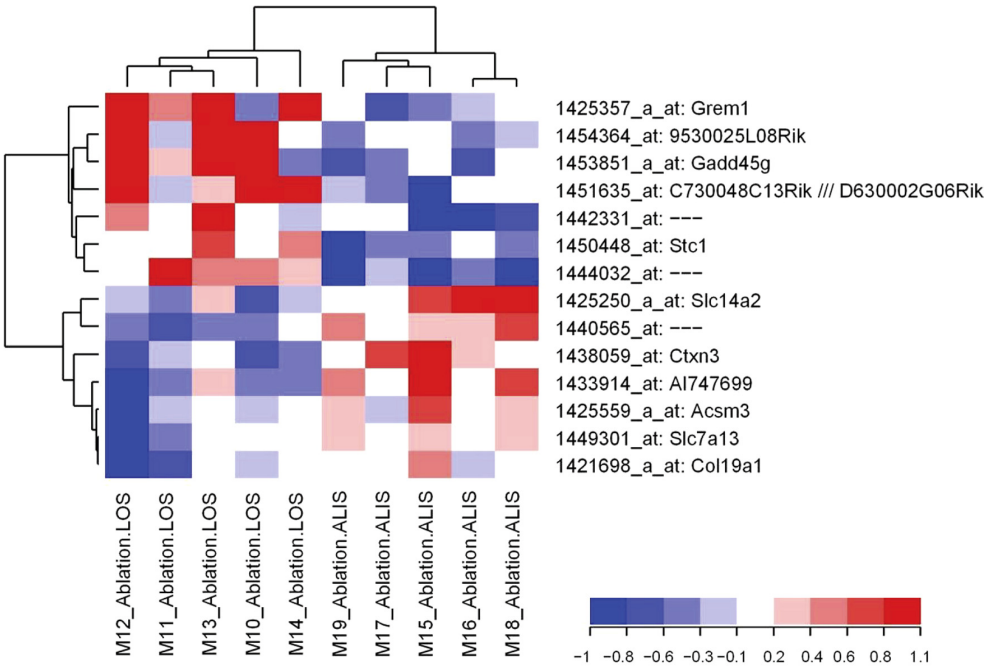

Fig. 5. Microarray analysis of renal gene expression comparing mice with renal ablation treated with either 500 mg/l losartan or 50 mg·kg^{-1}·day^{-1} aliskiren revealed significant regulation of 14 genes only. Clustering demonstrated a high intragroup relationship within each treatment group.

to AT_1 antagonism does not result in additional nephroprotection.

Measuring angiotensin fragments and discovering new ones have been described by us (17–19). In the present study, we measured ANG II levels by mass spectrometry and by ELISA and were unable to detect increased levels of ANG II and other fragments in mice treated with high-dose losartan. It has been suggested that the levels of ANG II and other fragments increase in species treated with angiotensin receptor blockers. However, it should be taken into account that angiotensinogen levels in mice are low (as opposed to in humans), so that increases in renin result in depletion of angiotensinogen as shown previously (30). Consequently, a rise in renin (as will always occur during RAS blockade) will not necessarily result in a rise in ANG II (28).

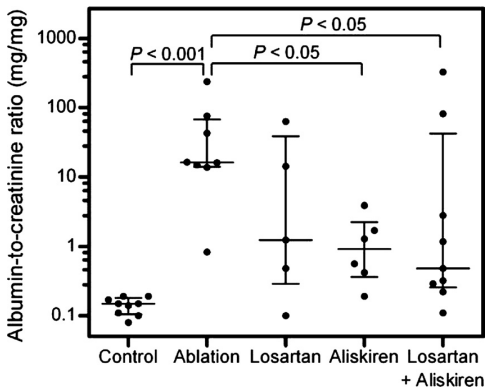

Fig. 6. Measuring albuminuria, a 6-wk combination therapy of losartan plus aliskiren in mice with chronic kidney disease due to renal ablation was not superior to both monotherapies.

Excellent nephroprotection by renin inhibition has been demonstrated in double transgenic rats carrying both the human renin and angiotensinogen gene as well as in diabetic rats overexpressing the mouse renin-2 gene (22, 33). However, since the overexpression of the renin gene causes end-organ damage, it is expected that aliskiren is protective. In fact, both transgenic models are based on high renin levels and are contrary to human CKD and renal ablation used in this study, where renin is downregulated.

Renin inhibition has been suggested to be superior to ACE inhibition or AT_1 antagonism in previous, probably less well-controlled studies (22). Moreover, the combination of AT_1 antagonism and renin inhibition has shown superior protection against renal fibrosis in models of diabetic nephropathy, unilateral ureter obstruction, and hypertensive renal injury in mice (8, 39–40). However, in none of these studies was RAS inhibition quantified as in the present study, nor did aliskiren monotherapy exceed low-dose treatment. Further studies also reported protective effects of renin inhibition. In some of those studies, however, the effects were compared vs. untreated animals (10, 12, 16, 21, 34).

Six-week administration of losartan and aliskiren significantly lowered albuminuria and glomerular injury. Both drugs did not reduce the increased interstitial volume caused by renal ablation. This is in line with our previous work demonstrating that RAS blockade decreases glomerular damage but is less efficient with regard to interstitial injury (13–14). Whether RAS blockade induced regression of

Table 3. *Double blockade*

Group	*n*	Mortality after Randomization	Blood Urea Nitrogen, mg/dl	Systolic Blood Pressure, mmHg	Renin RNA (relative expression)	Fibronectin RNA (relative expression)	Glomerular Sclerosis
Control	9	0	33.5 [27.5; 38.8]	95.0 [90.0; 100.0]	1.01 [0.76; 1.31]	1.05 [0.81; 1.22]	0.04 [0.02; 0.06]
Ablation	11	3	82.0 [74.5; 91.0]§	135.0 [123.8; 145.0]§	0.16 [0.09; 0.24]	5.69 [3.35; 6.61]§	0.74 [0.47; 1.20]§
Ablation+losartan	7	2	78.0 [66.8; 81.6]§	112.5 [105.0; 115.0]*	1.33 [0.82; 1.37]#	1.63 [1.32; 3.27]#	0.66 [0.49; 0.73]‡
Ablation+aliskiren	7	1	65.0 [64.3; 79.3]*§	100.0 [95.0; 100.0]†	1.26 [0.69; 1.44]	1.66 [1.44; 1.97]†	0.90 [0.48; 0.99]‡
Ablation+losartan+aliskiren	11	2	77.0 [72.3; 83.3]§	115.0 [90.0; 125.0]†	1.62 [1.41; 2.08]†	1.86 [1.30; 3.68]†	0.98 [0.62; 1.04]*‡

Values are expressed as the median [25th percentile; 75th percentile]. *$P < 0.05$ vs. ablation. †$P < 0.005$ vs. ablation. ‡$P < 0.05$ vs. control. §$P < 0.001$ vs. control.

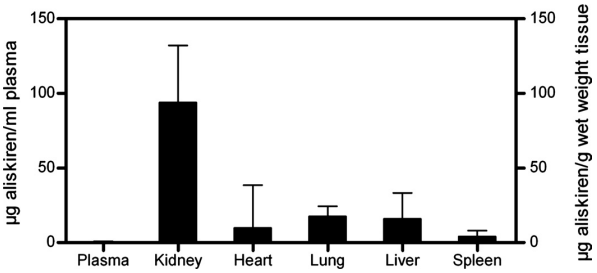

Fig. 7. After 6-wk treatment, plasma levels ($n = 6$) of renal-ablated mice treated with 50 mg·kg^{-1}·day^{-1} aliskiren were 0.60 μg/ml [0.50; 0.71]. In contrast, tissue levels ($n = 5$–6/group) were between 4.00 and 17.50 μg/g wet wt for heart, lung, liver, and spleen. The concentration of aliskiren in the kidney was 93.85 μg/g wet wt [67.70; 122.25], suggesting specific renal accumulation of aliskiren beyond extensive tissue distribution in various organs.

renal injury or delayed progression of CKD in this study remains elusive. Long-term RAS inhibition beyond 6 wk may resolve this issue. Aliskiren has been shown to accumulate in the kidney of rats (10). Feldman et al. (10) found a ~46-fold kidney-to-plasma ratio in rats treated with 10 mg·kg^{-1}·day^{-1} aliskiren (10). The present study validates and expands these observations. This is the first report in mice that aliskiren accumulates ~156-fold in the kidney compared with plasma. Moreover, we demonstrate for the first time a ~7- to 29-fold accumulation in the heart, lung, liver, and spleen. After withdrawal, aliskiren plasma levels dropped within 24 h to nondetectable, whereas a delayed decline over several days was found in the heart and kidney. Interestingly, this contrasts with plasma half-life of aliskiren in humans (~30–40 h after multiple dosing) (15), which was used to explain sustained reduced blood pressure after a missed dose and may counteract adverse effects due to imperfect adherence (5, 31). Interspecies variation of the pharmacokinetic entities of aliskiren thus need further evaluation.

The mechanisms for the accumulation of aliskiren are yet unknown. There is evidence that aliskiren accumulates in renin secretory granules, and, after exposure to renin-secreting cells, aliskiren-bound renin is released (26). In addition, Batenburg et al. (2) reported increased prorenin half-life in rat vascular smooth muscle cells after aliskiren binding. It is therefore likely that a part of the renal-accumulated aliskiren observed in our study is bound to renin within the juxtaglomerular cells. We propose that aliskiren accumulates ~7- to 29-fold in the tissue by a yet unknown mechanism possibly involving the (pro)renin receptor or the putative clearance receptor of renin, the M6P/IGF2-receptor (2). The higher renal accumulation may relate to the renin content of the kidney.

Although renal accumulation of aliskiren was only measured in the present study in healthy mice, we speculate that the renal aliskiren accumulation may cause prolonged antiproteinuric effects after treatment cessation in CKD mice, emphasizing its putative pathophysiological significance. The re-increase in albuminuria was significantly lower after withdrawal of aliskiren than of losartan. Due to the short plasma half-life of aliskiren we observed in mice, this effect was most likely independent of plasma concentrations. This

is in accordance with the recent observation of a prolonged antiproteinuric effect of aliskiren in rats (35). However, renal accumulation and prolonged efficacy after withdrawal may also be disadvantageous. Recently, the ALTITUDE trial, in which aliskiren was given on top of ACE inhibition and AT_1 antagonism to high-risk patients with diabetes and renal impairment, was prematurely terminated due to increased morbidity and side effects like hyperkalemia in the double blockade group. In cases of volume depletion by i.e., diarrhea, complete and long-lasting RAS blockade may aggravate acute renal failure. A similar observation was made in the double blockade group in the ONTARGET study (41).

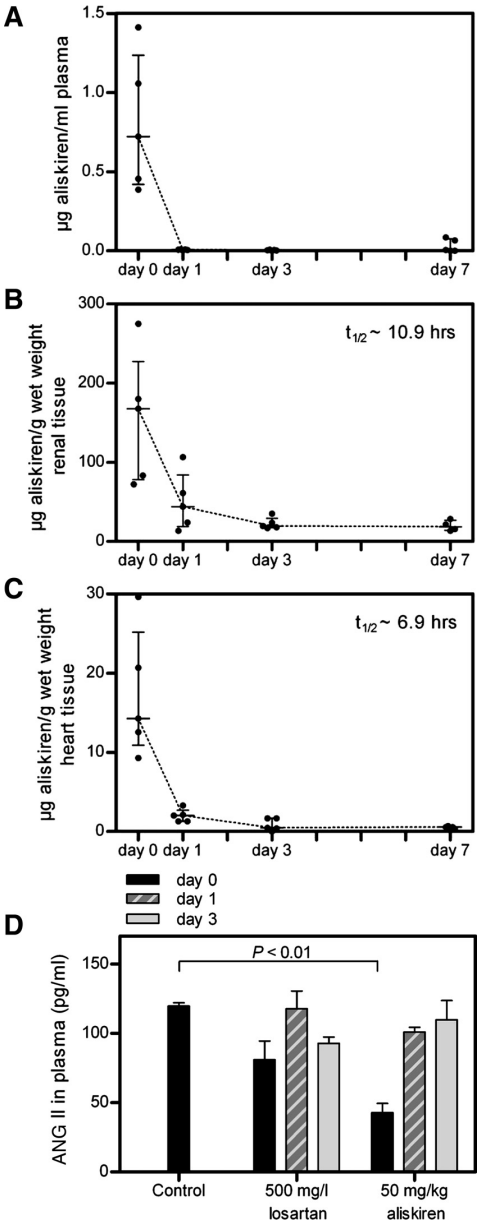

Fig. 8. Healthy FVB/N mice were treated with aliskiren for 14 days before treatment was withdrawn and mice were immediately euthanized (*day 0*) or 1, 3, or 7 days thereafter. Aliskiren was eliminated from the plasma within 24 h (*A*), but a decline was delayed from both kidney (*B*) and heart (*C*) tissue. For a clear arrangement, different scaling of the *y*-axis is used in the figure. Plasma ANG II levels measured by ELISA (n = 5/group) are shown in *D*. ANG II levels were decreased in aliskiren-treated mice and reappeared on *day 1* after cessation. Moreover, ANG II plasma levels were not increased in losartan-treated mice. Values are means ± SE.

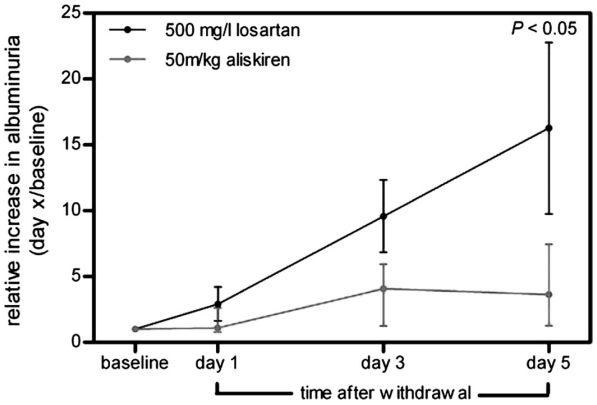

Fig. 9. Subsequent to a 6-wk treatment with aliskiren or losartan (n = 8 each) in mice with chronic kidney disease, cessation of therapy revealed a re-increase in albuminuria. This re-increase was significantly lower after withdrawal of aliskiren compared with withdrawal of losartan. Values are means ± SE.

In summary, RAS blockade at the level of renin and the AT_1 receptor, adjusted for equipotent dosages, offers similar nephroprotection in mice with CKD caused by renal ablation. This underscores the importance to target ANG II for nephroprotection. Combining renin inhibition and AT_1 antagonism did not exert additional effects. Finally, aliskiren accumulated ~156-fold in the kidney and ~7- to 29-fold in other organs compared with plasma, providing prolonged antiproteinuric effects.

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AUTHOR CONTRIBUTIONS

Author contributions: C.F., C.K., R.A.S., and U.O.W. provided conception and design of research; C.F., S.L., C.K., A.H., J.B., E.S., I.G., A.J.D., A.-R.F., H.v.G., V.J., and U.O.W. performed experiments; C.F., S.L., C.K., N.D., E.S., T.S., J.V., I.G., A.J.D., A.-R.F., H.v.G., V.J., G.N., and U.O.W. analyzed data; C.F., S.L., C.K., E.S., T.S., A.J.D., V.J., G.N., and U.O.W. interpreted results of experiments; C.F., J.V., and U.O.W. prepared figures; C.F. and U.O.W. drafted manuscript; C.F., S.L., C.K., E.S., T.S., J.V., A.J.D., and U.O.W. edited and revised manuscript; C.F., S.L., C.K., A.H., J.B., N.D., E.S., T.S., J.V., I.G., A.J.D., A.-R.F., H.v.G., V.J., R.A.S., G.N., and U.O.W. approved final version of manuscript.

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Aliskiren accumulation in the kidney: no major role for binding to renin or prorenin
Lange S, Fraune C et al. (2013) J Hypertens 31:713-719.

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Auf nachfolgender Seite finden sich eine Auflistung der Autoren und das Abstract dieser Veröffentlichung.

Lange S, Fraune C et al. (2013) J Hypertens 31:713-719

Titel: Aliskiren accumulation in the kidney: no major role for binding to renin or prorenin

Autoren: Sascha Lange[1,*], Christoph Fraune[1,*], Natalia Alenina[2], Michael Bader[2], A.H. Jan Danser[3], Anne-Roos Frenay[4], Harry van Goor[4], Rolf Stahl[1], Genevieve Nguyen[5], Edzard Schwedhelm[6] und Ulrich Otto Wenzel[1]

[1]III. Medizinische Klinik, Universitätsklinikum Hamburg-Eppendorf; [2]Max-Delbrück-Centrum für Molekulare Medizin, Berlin-Buch; [3]Institut für Pharmakologie, Erasmus Medical Center, Rotterdam, Niederlande; [4]Institut für Pathologie und Medizinische Biologie, Universitätsklinikum Groningen, Niederlande; [5]Collège de France, Paris, Frankreich und [6]Institut für Klinische Pharmakologie, Universitätsklinikum Hamburg-Eppendorf

[*]geteilte Erstautorenschaft

Abstract:

Background and objective: The antihypertensive effects of the direct renin inhibitor aliskiren last substantially longer after treatment withdrawal than expected based upon its plasma half-life. This may be attributable to drug accumulation in the kidney as recently shown in rats and mice. Since aliskiren binds to renin we examined in the present study whether this accumulation depends on the renin content of the kidney.

Methods: For this we measured the aliskiren concentration in the kidney of wild-type as well as AT1a receptor(-/-) and Ren1c(-/-) mice. AT1a receptor(-/-) mice overexpress renin due to the lack of angiotensin II-mediated negative feedback, whereas Ren1c(-/-) mice lack renal renin expression.

Results: Accumulation of aliskiren was found in the kidney of wild-type mice. However, renal accumulation was neither influenced by the overexpression nor by the absence of renin in the kidney. It was recently shown that the effects of aliskiren can be blocked by a handle region peptide, which inhibits the nonproteolytic activation of prorenin bound to the (pro)renin receptor. To investigate whether this putative (pro)renin receptor blocker in- fluences renal aliskiren accumulation, we administered the blocker in addition to aliskiren. No influence on renal aliskiren accumulation was observed.

Conclusion: These data confirm accumulation of aliskiren in the murine kidney and demonstrate that neither renin nor (pro)renin receptor-bound prorenin are major players in this process.

II. Darstellung der Publikationen

Inhibitoren des Renin-Angiotensin-Systems (RAS) gelten als pharmakotherapeutisches Mittel der Wahl bei chronischer Niereninsuffizienz, ihre Wirksamkeit ist durch diverse klinische Studien belegt (Jafar et al. 2003, Lewis et al. 2001). Die Überlegenheit von RAS-Inhibitoren gegenüber anderen Therapeutika wird im Kontext des RAS als Mediator renaler Endorganschäden jenseits der klassischen Funktion Blutdruckregulierung mittels Salz- und Volumenretention deutlich (Campbell 2004). Experimentell ermöglicht die Verwendung von RAS-Inhibitoren die pathophysiologische Bedeutung unterschiedlicher Angiotensinfragmente innerhalb des RAS genauer zu charakterisieren.
Ziel dieser Dissertation war unter tierexperimentellen Bedingungen im Modell der renalen Ablation die Bedeutung von RAS-Komponenten im Rahmen der chronischen Niereninsuffizienz unter Verwendung der RAS-Inhibitoren Aliskiren und Losartan zu untersuchen sowie die vermutete renale Akkumulation von Aliskiren unter pathophysiologischen Bedingungen zu prüfen. Die Ergebnisse dieser Dissertation wurden in zwei Publikationen (siehe I.) veröffentlicht und werden im Folgenden im weiterführenden Kontext erläutert.

Renale Ablation als Modell der chronischen Niereninsuffizienz

Die renale Ablation – meist und auch in den Experimenten der vorliegenden Arbeit als zweizeitige 5/6-Nephrektomie durch initiale 2/3-Nephrektomie und nachfolgende kontralaterale Uninephrektomie durchgeführt – ist ein seit Jahrzehnten etabliertes Modell zur Studie der chronischen Niereninsuffizienz (Bradford 1899, Morrison und Howard 1966). Die Wirksamkeit bedeutender therapeutischer Strategien bei chronischen Nierenerkrankungen wie Hemmung des Angiotensin-Converting-Enzymes (ACE) und Proteinrestriktion konnte ursprünglich an renal abladierten Ratten nachgewiesen werden (Anderson et al. 1986, Brenner et al. 1982, Meyer et al. 1985). Basis des Modells ist die partielle chirurgische Reduktion von funktionsfähigem Nierengewebe – durch kompensatorische Hyperfiltration der verbliebenen Nephrone entwickelt sich daraus mittelfristig ein progressiver Nierenschaden im Sinne einer Maladaptation (Kren und Hostetter 1999). Funktionell und histomorphologisch entspricht die renale Ablation der chronischen Niereninsuffizienz beim Menschen hinreichend (Metcalfe 2007).

Renale Suszeptibilität der FVB/N-Mauslinie

Für die Experimente der vorliegenden Arbeit wurden FVB/N-Mäuse verwendet, da diese suszeptibel gegenüber renaler Ablation sind – im Gegensatz zu Mäusen der C57bl6-Mauslinie und verwandter Linien, welche trotz massiver Ablation kaum messbare Surrogatparameter renaler Schädigung oder gar histomorphologische Veränderungen offenbaren (Benndorf et al. 2009). Das Ausmaß des in dieser Arbeit operativ induzierten Nierenschadens lässt sich anhand einiger Parameter abschätzen: massive Proteinurie (Fraune et al. 2012, Abbildung 1), Versagen der exkretorischen Nierenfunktion mit Urämie (Fraune et al. 2012, Tabelle 1 und Tabelle 2), moderate arterielle Hypertonie (Fraune et al. 2012, Tabelle 2) und histomorphologisch glomeruläre Läsionen sowie Tubulusatrophie mit interstitieller Fibrose im Rahmen der Perpetuierung des Schadens (Fraune et al. 2012, Abbildung 3). Der induzierte renale Schaden der FVB/N-Mäuse liegt also im nephrotischen Bereich – dies unterstreicht die Übertragbarkeit entsprechender Experimente auf den klinischen Kontext.

Abgrenzung der renalen Ablation zu konkurrierenden Mausmodellen

In der vorliegenden Arbeit wurde die pathophysiologische Bedeutung von Angiotensinfragmenten für die Progression der chronischen Niereninsuffizienz untersucht. Dazu wurde an renal abladierten Mäusen ein Wirksamkeitsvergleich der beiden RAS-Inhibitoren Aliskiren und Losartan durchgeführt, welche an entgegengesetzten Abschnitten innerhalb der RAS-Kaskade eingreifen. Für diese und ähnliche Fragestellungen bedarf es eines Modells, dessen pathophysiologische Veränderungen innerhalb des RAS denen der chronischen Niereninsuffizienz hinreichend entsprechen.

In diesem Zusammenhang ist die verminderte renale Reninexpression nach renaler Ablation entscheidend (Fraune et al. 2012, Abbildung 4A). Diese entspricht der niedrigen Plasmareninaktivität einer chronischen Niereninsuffizienz konsekutiv Salz- und Flüssigkeitsretention, die in Anbetracht der hervorragenden Wirksamkeit von RAS-Blockern bei chronischen Nierenerkrankungen zunächst überraschen mag (Campbell 2004). Dieses scheinbare Paradoxon lässt sich aus aktueller Sicht – ähnlich dem kardialen *tissue remodeling* einer chronischen Herzinsuffizienz – durch gewebeabhängige, also lokale RAS begründen, welche die pathogenetischen Mechanismen der Organschädigung unmittelbar katalysieren oder gar perpetuieren. Beispielsweise ist die profibrotische Wirkung von Angiotensin (Ang) II über die Induktion von *transforming growth factor beta* (TGF-β) lange bekannt (Kagami et al. 1994).

Die Verwendung anderer klassischer Tiermodelle aus dem Gebiet Hypertonie und Nephropathie – zum Beispiel für humanes Angiotensinogen und humanes Renin doppelt-transgener

Ratten (dTGR) (Ganten et al. 1992) oder TGR(mRen2)27-Ratten, die murines *Ren2* überexprimieren (Mullins et al. 1990) – ist bei der Beurteilung der biologischen Funktion von RAS-Komponenten im Kontext von chronischen Nierenerkrankungen zumindest fragwürdig: diese Modelle bilden die real-pathophysiologischen Prozesse einer chronischen Niereninsuffizienz beim Menschen nicht adäquat ab. Beide Modelle basieren auf einer genetischen Überexpression von RAS-Komponenten ohne klinisches Korrelat; so ist die Attenuierung einer Gewebeschädigung durch Renininhibition in einem Modell, dessen pathogenes Rückgrat die Überexpression von Renin darstellt, zu erwarten (Pilz et al. 2005). Als *condicio sine qua non* für eine adäquate Beurteilung der pharmakotherapeutischen Möglichkeiten im RAS ist unter klinisch relevanten Bedingungen vielmehr ein Modell erforderlich, welches die real-pathophysiologischen Mechanismen imitiert.
Die renale Ablation in FVB/N-Mäusen erfüllt diese Anforderung hinreichend, auch wenn die experimentelle Ablation von Nierengewebe natürlich nicht den gewöhnlichen ätiologischen Stimulus einer chronischen Nierenerkrankung beim Menschen darstellt. Bezüglich der Aggravation der ursprünglichen Schädigung ist der auslösende Faktor jedoch unwesentlich, da die Progression chronischer Nierenerkrankungen vornehmlich dem fortschreitenden Verlust funktionsfähiger Nephrone und sekundär bedingter tubulointerstitieller Veränderungen unterliegt (Hodgkins und Schnaper 2012), unbedeutend ob dies durch vaskuläre Ursachen, eine chronische Pyelonephritis, chronische Glomerulonephritis oder experimentell durch Ablation verursacht wurde (Morrison und Howard 1966).

RAS-Inhibition als Methode zur Dechiffrierung des RAS

Neben der oben genannten Induktion von TGF-β sind weitere parakrine Funktionen des RAS im Rahmen zytopathischer Vorgänge wie beispielsweise Aktivierung von *nuclear factor-kappa B* (NFκB) mit Rekrutierung von Entzündungszellen, somit jenseits arterieller Vasokonstriktion und Natriumhomöostase, beschrieben (Ruiz-Ortega et al. 2006, Ruiz-Ortega et al. 1998). Das RAS ist mannigfaltiger als dessen langjährig reduzierte konzeptionelle Betrachtung als plasmatische Kaskade zur linearen proteolytischen Degradation von Angiotensinogen zu Ang II vermuten ließ. Zunehmend werden immunmodulatorische Effekte von Angiotensinfragmenten aufgedeckt: humane T-Lymphozyten exprimieren ein funktionsfähiges zelluläres RAS (Jurewicz et al. 2007), sodass eine RAS-Blockade das numerische Verhältnis von regulatorischen gegenüber effektorischen T-Zellen verändern kann (Platten et al. 2009). Letztere sind eventuell unmittelbar an der Genese von Ang II-vermittelten hypertensiven Schäden beteiligt (Guzik et al. 2007).

Die Verwendung verschiedener RAS-Inhibitoren ermöglicht das RAS gezielt zu untersuchen um so die pathophysiologische Bedeutung einzelner RAS-Komponenten besser zu erfassen. Im Rahmen dieser Arbeit wurde dazu das Konzept der vollständigen RAS-Blockade durch den direkten Renininhibitor Aliskiren verfolgt und außerdem eine RAS-Inhibition durch den Angiotensin II Typ 1 (AT_1)-Rezeptorblocker Losartan durchgeführt.

Durch Aliskiren kann eine vollständige Suppression der RAS-Aktivität (trotz reflektorischen Anstiegs der Plasmareninkonzentration) erreicht werden, da mit Renin die initiale Protease, somit der geschwindigkeitsbestimmende Reaktionsschritt des RAS, inhibiert wird (Lu et al. 2008). Dies wird durch eine stöchiometrische Betrachtung deutlich: zwar vermag die plasmatische Angiotensinogenkonzentration aufgrund ihrer Nähe zur Michaeliskonstante (K_m) von Renin die Enzym-Substrat-Reaktion zur Bildung von Ang I theoretisch zu beeinflussen, da jene Konzentration unter den meisten pathophysiologischen Bedingungen jedoch konstant ist, und da Ang I im Plasma praktisch unmittelbar und vollständig durch das endotheliale ACE zu Ang II degradiert wird, ist die aktive Menge von Renin im Plasma Determinante der Generierung von Ang II und entsprechend verwandter Fragmente (Atlas 2007, Ayers 1967, Klett und Granger 2001). Durch ausreichend starke Dosierung von Aliskiren lassen sich daher sämtliche Angiotensinfragmente supprimieren; entsprechend konnten in dieser Arbeit weder Ang I, noch Ang II noch Ang (1-9) während Renininhibition massenspektrometrisch im Plasma nachgewiesen werden (Fraune et al. 2012, Abbildung 1C).

Losartan hingegen blockiert spezifisch die Wirkung von Ang II am AT_1-Rezeptor. Durch Disinhibition des negativen Feedbacks von Ang II auf die Renin-sezernierenden Zellen des juxtaglomerulären Apparats ist hier ein kompensatorischer Anstieg der Konzentration von Renin, Ang I und nachgeschalteter Angiotensinfragmente zu erwarten. Erstaunlicherweise konnte in dieser Arbeit keine wesentliche Veränderung von Ang I, Ang II und Ang (1-9) nach Gabe von Losartan beobachtet werden (Fraune et al. 2012, Abbildung 1C); möglich erscheint diesbezüglich eine Substratdepletion von Angiotensinogen, da dessen Konzentration in der Maus nicht – wie oben für den Menschen beschrieben – im Bereich der K_m von Renin, sondern deutlich darunter liegt. Dies könnte eine gesteigerte Generierung von Angiotensinfragmenten während einer AT_1-Blockade in der Maus verhindern.

Zusammenfassend ist der Einfluss dieser beiden RAS-Inhibitoren auf das RAS entgegengesetzt: Der Renininhibitor Aliskiren hebt die proteolytische Aktivität von Renin auf und vermindert so die Konzentration von Angiotensinfragmenten, nach AT_1-Blockade durch Losartan sind letztere dagegen beim Menschen erhöht und in den Experimenten dieser Arbeit in der Maus zumindest auf unverändertem Niveau (Burnier et al. 1995). Beiden Substanzen gemeinsam ist – wenn auch durch unterschiedliche Mechanismen hervorgerufen – die geminderte Wichtung von Ang II im RAS. Durch den Vergleich der nephroprotektiven Effekte

von Aliskiren und Losartan nach renaler Ablation lässt sich unter der Voraussetzung einer äquipotenten Dosierung (Fraune et al. 2012, Abbildung 1) daher die pathophysiologische Bedeutung von Ang II in diesem Modell untersuchen.

Angiotensin II als Haupteffektor renaler Schädigung

Unter äquipotenter Dosierung im Sinne der Inhibition des RAS wurde in dieser Arbeit der nephroprotektive Effekt von Aliskiren, Losartan und derer kombinierten Therapie an renal abladierten Mäusen verglichen. Sowohl für Aliskiren als auch für Losartan wurde ein substantieller nephroprotektiver Effekt nachgewiesen, vor allem die Quantifizierung der Albuminurie offenbarte eine hochsignifikante Reduktion gegenüber unbehandelten nierenkranken Mäusen (Fraune et al. 2012, Abbildung 2A). Darüber hinaus bestand nach sechswöchiger Therapie eine signifikante Reduktion des histologischen Schadens (Fraune et al. 2012, Abbildung 3), der Expression von Fibrosemarkern (Fraune et al. 2012, Abbildung 4C und D) sowie des systolischen Blutdrucks (Fraune et al. 2012, Tabelle 2) jeweils gegenüber unbehandelten nierenkranken Mäusen. Für keinen der analysierten Parameter wurde jedoch ein signifikanter Unterschied zwischen beiden Therapiegruppen selbst festgestellt. Durch Microarray-Analyse von über 34000 Gentranskripten wurden lediglich 14 signifikant unterschiedlich regulierte Transkripte zwischen Aliskiren- und Losartantherapie identifiziert, die keinem gemeinsamen biologischen Prozess zugeordnet werden konnten (Fraune et al. 2012, Abbildung 5). Dies bekräftigt, dass der nephroprotektive Effekt beider Pharmaka kongruent über ihren jeweiligen Einfluss auf die Genese beziehungsweise die Wirkung von Ang II vermittelt wird und weniger von anderen Angiotensinfragmenten abhängt. Damit unterstützen die Ergebnisse dieser Arbeit das gegenwärtige Konzept von Ang II als maßgeblichen Mediator RAS-vermittelter renopathischer Effekte bei chronischen Nierenerkrankungen.
Obwohl die nephropathischen Effekte des RAS also maßgeblich Ang II-vermittelt sind, ist eine biologisch relevante Wirkung anderer Angiotensinfragmente oder Rezeptoren jenseits Ang II und seiner Rezeptoren durch diese Arbeit letztlich nicht ausgeschlossen. Schließlich ist denkbar, dass einige Angiotensinfragmente entgegen gesetzte, nephropathische und nephroprotektive Effekte vermitteln, die sich bei ganzheitlicher Betrachtung des RAS maskieren und als Nettoeffekt in der vorliegenden Untersuchung somit nicht ins Gewicht fallen. Etwa ist möglich, dass aus therapeutischer Sicht kongruente Effekte des mas- oder AT_2-Rezeptors während AT_1-Antagonismus durch nephropathische Effekte des (Pro)Reninrezeptors ((P)RR) unentdeckt blieben. Pharmakologische Agonisten und Antagonisten der genannten Rezeptoren sind entwickelt (Ichihara et al. 2004, Wan et al. 2004,

Wiemer et al. 2002) und ermöglichen eine gezieltere Untersuchung dieser Faktoren als in der vorliegenden Arbeit.
Zusätzlich zu den Therapien mit Aliskiren oder Losartan wurde auch die entsprechende duale Therapie mit Aliskiren in Kombination mit Losartan durchgeführt und mit beiden Monotherapien verglichen. Hier wurde keine Überlegenheit der dualen RAS-Inhibition festgestellt (Fraune et al. 2012, Abbildung 6 und Tabelle 3). Dies ist vor allem unter der Berücksichtigung des deutlichen Anstiegs der Plasmarenin*aktivität* unter AT_1-Blockade von Interesse, welche durch Koadministration eines Renininhibitors verhindert werden kann. Die Auswirkungen erhöhter Plasmarenin*aktivität* (unter AT_1-Inhibition) und Plasmarenin*konzentration* (unter Renininhibition) bleiben im Kontext des kürzlich entdeckten (P)RR, welcher die katalytische Aktivität von Renin und dessen Präkursors Prorenin steigert und zusätzlich enzymunabhängige, rezeptorvermittelte Effekte transduziert (Nguyen et al. 2002), weiterhin enigmatisch. Eine signifikante Regulation des (P)RR in der Niere, wie in anderen Arbeiten vorbeschrieben, konnte in der vorliegenden Arbeit nicht bestätigt werden (Feldman et al. 2008, Fraune et al. 2012, Abbildung 4B, Nguyen et al. 2002).

Akkumulation von Aliskiren in der Niere

In der vorliegenden Arbeit wurde zur vollständigen RAS-Blockade Aliskiren verwendet. Dieses ist der Prototyp einer neuen Substanzklasse von oral verfügbaren direkten Renininhibitoren. Im Vergleich zu anderen Antihypertensiva fällt Aliskiren durch eine lange Plasmahalbwertszeit von ca. 40 Stunden sowie durch renale Akkumulation auf (Feldman et al. 2008). Sowohl in klinischen als auch experimentellen Arbeiten wurde eine prolongierte Wirkung nach Ende einer Pharmakotherapie mit Aliskiren nachgewiesen (Düsing et al. 2012, Palatini et al. 2010, Rakušan et al. 2010). Dies könnte einem durch geringe Pharmakoadhärenz von Patienten verursachten abgeschwächten Therapieerfolg mit der Substanz entgegenwirken, denkbar ist allerdings auch eine erhöhte Rate von unerwünschten Arzneimittelreaktionen.
In dieser Arbeit konnten jene Beobachtungen auf FVB/N-Mäuse übertragen werden – es wurde eine starke Akkumulation von Aliskiren in der Niere (ca. 160-fach) und in anderen Organen (7 bis 30-fach) beobachtet (Fraune et al. 2012, Abbildung 7), ebenso wurde ein prolongierter antiproteinurischer Effekt von Aliskiren nach Therapieende aufgezeigt (Fraune et al. 2012, Abbildung 9). Zudem wurde die Plasma- und Gewebehalbwertszeit von Aliskiren untersucht und erstmals für die Maus beschrieben (Fraune et. al 2012, Abbildung 8A-C).
Da die plasmatische Halbwertszeit von Aliskiren in dieser Arbeit deutlich weniger als 24 Stunden betrug, ist die beobachtete prolongierte Wirkung vermutlich unabhängig der Plas-

makonzentration (Fraune et al. 2012, Abbildung 8A). Dies stärkt die These der Gewebeakkumulation von Aliskiren als Ursache der verlängerten Wirkung maßgeblich.
Ein Zusammenhang zwischen der renalen Reninkonzentration und der Akkumulation von Aliskiren in der Niere ist denkbar, denn Aliskiren bindet an Renin und dieses wird in der Niere stark exprimiert. Eine Kolokalisation von Aliskiren und Renin wurde bereits *in vitro* beschrieben (Krop et al. 2008). Im Rahmen dieser Arbeit wurden weitere Experimente *in vivo* durchgeführt um so einen möglichen ursächlichen Zusammenhang zwischen Renin und der Gewebeakkumulation von Aliskiren aufzudecken (Lange et al. 2013).
Durch knockout-Mauslinien spezieller RAS-Komponenten ist die Konzentration von Renin in der Niere manipulierbar: AT1a-Rezeptor-Knockout ($AT1a^{-/-}$)-Mäuse exprimieren Renin stark über, wohingegen Ren1c-Knockout ($Ren1c^{-/-}$)-Mäuse kein Renin haben (Lange et al. 2013, Abbildung 1). In Experimenten dieser Arbeit wurden die renalen Spiegel von Renin und Aliskiren nach entsprechender Therapie bestimmt und verglichen. Weder ein erhöhter Aliskirenspiegel in $AT1a^{-/-}$-Mäusen noch eine signifikant verminderte Konzentration oder gar vollständige Depletion von Aliskiren in den Nieren von $Ren1c^{-/-}$-Mäusen wurden gefunden (Lange et al. 2013, Abbildung 4B), sodass Renin als Determinante der renalen Aliskirenkonzentration unwahrscheinlich ist.
Möglich erscheint eine Beteiligung des kürzlich entdeckten (P)RR, da dieser Prorenin in offener Konformation als auch Renin bindet und anschließend internalisiert. Somit könnte sich (Pro)Renin-gebundenes Aliskiren mittels des (P)RR im Gewebe anreichern. Durch die Gabe eines sogenannten *handle region peptides* (HRP), welches dem Prosegment von Prorenin ähnelt, lässt sich die Interaktion von (Pro)Renin mit dem (P)RR jedoch unterbinden (Ichihara et al. 2004). Ein Einfluss des HRP auf den Gewebespiegel von Aliskiren während der Koadministration beider Substanzen wurde durch die Experimente dieser Arbeit allerdings nicht festgestellt (Lange et. al 2013, Abbildung 5). Damit ist auch (P)RR-gebundenes (Pro)Renin als Ursache der renalen Akkumulation von Aliskiren unwahrscheinlich. Der Mechanismus der Gewebeakkumulation von Aliskiren bleibt weiterhin unklar.

Zusammenfassung

Im Rahmen dieser Arbeit wurde das Modell der renalen Ablation in der FVB/N-Mauslinie erfolgreich auf relevante Forschungsaspekte der chronischen Niereninsuffizienz angewendet. Durch gezielte pharmakologische Manipulation im RAS mittels der RAS-Inhibitoren Aliskiren und Losartan konnte die Bedeutung von Ang II als entscheidender Mediator des RAS für den renalen Schaden identifiziert werden. AT_1-Antagonismus mit Losartan und Renininhibition mit Aliskiren sind bei äquipotenter RAS-Inhibition gleichermaßen nephropro-

tektiv. Bei Ausdosierung der Monotherapie erzielt eine Doppelblockade keinen weiteren Benefit.

Aus pathophysiologischer Sicht bestätigt diese Arbeit die paradigmatische Betrachtung von Ang II als kardinalen Mediator RAS-vermittelter Schäden; es unterliegt weitergehenden Experimenten die pathophysiologische Bedeutung anderer Angiotensinfragmente aufzuklären. Die beobachtete Akkumulation von Aliskiren in der Niere erscheint aus therapeutischer Sicht wegen der verlängerten antiproteinurischen Wirkung zunächst vielversprechend, vermag jedoch auch negative Effekte zu vermitteln. Es konnte eindeutig gezeigt werden, dass Renin nicht die Ursache der renalen Akkumulation von Aliskiren ist. Auch der (P)RR spielt dabei keine Rolle. Der Mechanismus der Gewebeakkumulation bleibt unklar.

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V. Erklärung des Eigenanteils

Folgende Anteile der vorliegenden Arbeit basieren auf meiner unmittelbaren (Mit-)Arbeit:

- Versuchsdesign, Versuchsplanung und Diskussion der Daten: gemeinsam mit Prof. Dr. Ulrich Wenzel
- Operative Eingriffe: gemeinsam mit Dr. Robin Schmidt-Haupt und Dr. Christian Krebs
- Betreuung der Mäuse während der Versuche: gemeinsam mit der Versuchstierhaltung des UKE
- Gewinnung von Urinproben mittels Stoffwechselkäfigen
- tail-cuff-Blutdruckmessungen: gemeinsam mit Dr. Christian Krebs
- Durchführung von RT-PCR und ELISA zur Analyse der Parameter nach Anleitung von Stefan Gatzemeier
- Mikroskopische Auswertung der histologischen Schnitte
- Planung und Durchführung der statistischen Analyse nach Beratung am Institut für Medizinische Biometrie und Epidemiologie am UKE durch Lena Herich
- Schreiben des Manuskripts, Erstellen der Abbildungen und Tabellen sowie Veränderungen des Manuskripts im Rahmen des Reviewprozesses: gemeinsam mit Prof. Dr. Ulrich Wenzel

Die Durchführung nachfolgender Anteile der vorliegenden Arbeit basiert *nicht* auf meiner unmittelbaren Arbeit, wohl aber die Auswertung der daraus generierten Daten sowie deren Interpretation und Diskussion:

- Dosiswirkungskurve: cand. med. Alexandra Hölzel und Dr. Jana Baucke
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VI. Curriculum vitae

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VII. Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich diese Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.

Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.

Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Christoph Fraune