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Involvement of renal ACE activity in proteinuria-associated renal damage in untreated and treated adriamycin nephrotic rats

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Abstract

Proteinuria is assumed to play a pathogenetic role in progressive renal damage. Angiotensin-converting enzyme (ACE) inhibition reduces proteinuria and provides renoprotection. This suggests that ACE activity might play a pathogenetic role in the development of proteinuria-induced renal structural damage. We investigated this hypothesis in untreated and treated established adriamycin nephrosis, a model of proteinuria-induced renal damage.

In a time-course experiment, the development of renal structural damage in untreated adriamycin nephrotic rats was paralleled by a significant rise in renal ACE activity. Moreover, on cross-sectional analysis, a consistent positive correlation between renal, but not plasma, ACE activity and proteinuria, focal glomerulosclerosis and interstitial injury was present. Notably, these associations were present, not only in the untreated condition, but also during intervention with either ACE inhibition or AT₁-receptor antagonism. Interestingly, we found that higher renal ACE activity is associated with more severe renal damage for a given amount of proteinuria, suggesting that renal ACE activity may be either a permissive or a promoting factor in the processes by which proteinuria eventually leads to renal structural damage. This relationship was abolished by renin-angiotensin system (RAS)-blockade, suggesting that RAS-mediated effects are involved in the relationship between renal ACE activity and proteinuria-induced renal damage.

In conclusion, in untreated as well as treated adriamycin nephrotic rats, renal ACE activity is closely associated with renal outcome. This association appears to be independent of the specific mode of blockade of the RAS. Renal ACE activity is a consistent marker of individual differences in proteinuria-associated renal damage: further studies are needed to investigate a possible pathogenetic role in renal damage.

Introduction

Proteinuria is assumed to play a pathogenetic role in progressive renal damage.¹ This hypothesis is supported by the consistent predictive value of the severity of proteinuria for subsequent progressive renal damage in human and experimental renal disease.^{2,3} Moreover, during intervention, reduction of proteinuria predicts the efficacy of protection against ongoing renal damage.^{4,6} The

mechanisms of proteinuria-induced renal damage, however, are incompletely understood.

Angiotensin-converting enzyme (ACE) inhibition reduces proteinuria and protects against progressive renal damage.^{2,6,7} This suggests that ACE activity might play a pathogenetic role in the development of proteinuria-associated renal structural damage. This assumption is supported by recent experimental data, demonstrating upregulation of renal tubular ACE in the protein-overload model of proteinuria in the rat.⁸ In the present study, therefore, we investigated the possible pathogenetic role of renal ACE activity in proteinuria-induced renal damage. To this purpose, we used the model of adriamycin-induced nephrotic syndrome, a model of proteinuria-induced renal structural damage. In this model, we investigated renal ACE activity, and its association with proteinuria and renal structural damage during the natural course, and during intervention with antiproteinuric treatment using an ACE inhibitor (ACE-I) or an AT₁-receptor blocker (ARB).

Methods and design

The protocol was approved by the Committee for Animal Experiments of the University of Groningen, The Netherlands. The study was conducted in accord with the NIH Guide for the Care and Use of Laboratory Animals. Male Wistar rats (Hsd.Cpd.Wu; Harlan Inc., Zeist, The Netherlands) were studied. Throughout the study, the rats were housed in a temperature-controlled room with a 12-hour light-dark cycle. All animals were fed a low salt diet (0.05% salt, 20% protein, Hope Farms Inc., Woerden, The Netherlands) *ad libitum* from one week before study entry and received daily fresh tap water *ad libitum*. Once a week during the entire protocol, urine was collected during a 24-hour stay in metabolic cages with free access to food and water, to determine proteinuria. Intake of water and food were measured during the 24-hour stay in the metabolic cage. Body weight and systolic blood pressure (BP) were measured weekly. Animals were trained before study entry to become accustomed to handling, metabolic cages and BP measurements.

Induction of nephrosis

Proteinuria, systolic BP (SBP) and intake of water

and food were measured once before inducing the nephrosis. Subsequently, rats were anaesthetised with isoflurane/O₂/N₂O and 1.5 mg/kg adriamycin was injected into the penis vein. This dose of adriamycin was chosen to induce a modest and stable proteinuria, as confirmed by prior experiments in our laboratory by Wapstra and later modified by De Boer.^{9,10} In this model, proteinuria stabilises six weeks after injection of adriamycin.

Protocol A: time-course of renal ACE activity in adriamycin nephrosis

Sixteen healthy rats were randomised to two groups of eight rats each. In one group, adriamycin nephrosis was induced as described above. After stabilisation of proteinuria, i.e. six weeks after induction of the nephrosis, the lower pole of the left kidney was surgically resected and harvested for determination of renal ACE activity. The other group served as healthy controls. These animals were injected with saline and did not undergo renal biopsy. The rats in this experiment did not receive antiproteinuric treatment and were terminated at study week 12, and kidneys harvested for determination of focal glomerulosclerosis and renal ACE activity.

Protocol B: renal ACE activity and renal outcome in untreated and treated adriamycin nephrotic rats

In this experiment, nephrosis was induced in 45 rats. After stabilisation of proteinuria, i.e. six weeks after induction of the nephrosis, these rats were stratified, according to the mean of the proteinuria in weeks five and six, into three groups (each group n=15). One group was treated with the ACE inhibitor, (ACE-I) lisinopril 75 mg/l drinking water (ACE-I treated rats), another group with the ARB, L 158,809, 100 mg/l drinking water (ARB treated rats) and the third group with vehicle (untreated rats). Treatment was continued for six weeks, i.e. until termination. At week 12, all rats were terminated. The dose of lisinopril, based on previous studies in our laboratory, was chosen to yield the maximal antiproteinuric effect.² The chosen dose of ARB has an equipotent antiproteinuric efficacy in the adriamycin model and was similar to that used earlier by Wapstra in the same model.¹¹

Termination

In both experiments, all rats were sacrificed under anaesthesia with isoflurane/O₂/N₂O and blood was collected by puncture of the abdominal aorta for determination of plasma ACE activity at study week 12. Subsequently, the kidneys were perfused with saline and harvested for histological examination and determination of renal ACE activity and ACE mRNA. Tissue processing was performed as described earlier by Van Goor *et al.*¹²

Measurements

Proteinuria

In both experiments, proteinuria was determined by the Biuret® method.

Blood pressure

In the second experiment, SBP was measured in conscious rats with an automated multichannel system, using tail cuffs and photoelectric sensors to detect the tail pulse (Apollo 179, IITC Life Science, Woodland Hills, CA, USA). The rats were placed in the test chamber in restrainers while temperature was regulated and maintained at 28–29°C. During each measurement session, three measurements were recorded for each animal. The value for SBP was taken as the mean of these three recordings.

Morphology

In the second experiment, the degree of focal glomerulosclerosis was scored semiquantitatively. Focal glomerulosclerosis was scored as positive when mesangial expansion, mesangial cellularity, adhesion formation and capillary obliteration were present in one segment. If 25% of the glomerulus was affected a score of one was given, 50% was scored as two, 75% as three and 100% as four. In both kidneys of each animal, 100 randomly-chosen glomeruli were examined, and their scores were added. The theoretical maximum score was therefore 400. The mean of the scores of both kidneys is given as the focal glomerulosclerosis score.¹³ Moreover, interstitial injury was scored semiquantitatively on a scale of 0–3, regarding tubular dilatation and/or atrophy, interstitial fibrosis, and inflammatory cell infiltrates. The mean of the scores of both kidneys is given as the score for interstitial injury.

ACE activity

The method used for determination of ACE activity is the one previously described by Hirsch *et al.*¹⁴ and Buikema *et al.*¹⁵ Renal tissue was homogenised in a 50 mM K₂PO₄ homogenate buffer at pH 7.5. Subsequently, 100 µl of the diluted sample was pipetted into a 0.5 M K₂PO₄ buffer. As substrate, 100 µl of 12.5 mM Hip-His-Leu (Sigma), which is converted by ACE into the bipetide His-Leu, was added and incubated at 37°C for exactly 15 minutes. In this amount, the substrate is present in excess and thus not rate-limiting. The conversion of the substrate was stopped by adding 1.45 ml of 280 mM NaOH. Subsequently, 100 µl of 1% phthaldialdehyde was added. This adheres to the formed bipetide. The amount of tagged His-Leu was fluorimetrically determined at an excitation wavelength of 364 nm and an emission wavelength of 486 nm. This yields a measure of the amount of His-Leu generated in the sample. In blank samples, conversion of the substrate was prevented by prior addition of NaOH. To further ensure that no substrate would convert in the blank samples, the substrate was added only after the incubation period.

Tissue ACE mRNA

Renal tissue was homogenised. Subsequently, a semi-quantitative polymerase chain reaction (PCR) was performed to determine the expression of the ACE gene in this tissue (ACE mRNA). During this reaction, the mRNA of the ACE gene and the

Table 1 Group characteristics at the end of protocol B

	Body weight (gram)	Proteinuria (mg/day)	Change of proteinuria (% compared to baseline)	Systolic blood pressure (mmHg)	FGS-score (0-400)	Interstitial injury-score (0-3)
Untreated	436 (368-478)	672 (523-1001)	0 (-18-+26)	138 (113-149)	61 (14-98)	0.5 (0.0-1.0)
ACE-inhibitor	398 (357-425)*	113 (52-311)*	-85 (-91- -62)*	88 (67-113)*	20 (10-67)	0.0 (0.0-1.5)
ARB	435 (407-452)	170 (62-691)*	-74 (-85- -44)*	105 (82-133)*	20 (10-78)	0.0 (0.0-1.5)

Median and 95% confidence interval of the median. FGS-score = focal glomerulosclerosis-score
ACE = angiotensin-converting enzyme; ARB = AT₁-receptor blocker; * p<0.05 versus untreated animals

mRNA of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were coamplified in a single PCR reaction. A 50 µl PCR reaction mixture contained 1.0 units Taq polymerase (Eurogentec, Belgium), 5 µl of the supplied buffer, 40 nmol deoxynucleosidetriphosphate, 25 mM MgCl₂, 1 µl of cDNA mixture and 40 pmol of both gene-specific primers (Genbank Ac. numbers U03708 and AF106860 for ACE and GAPDH respectively). Temperature cycling was performed in 0.5 ml thin-walled tubes in a thermal cycler (DNA thermal cycler, Perkin Elmer, USA), using a protocol of 30 cycles of one minute denaturation at 94°C, one minute annealing at 56°C and one minute extension at 72°C. PCR products were separated on a 1.5% agarose gel by electrophoresis and stained with ethidium bromide. The gels were quantified by densitometry. The relative expression levels of ACE are expressed relative to GAPDH PCR products and normalised for the untreated adriamycin nephrotic rats.

Data analysis

Data are expressed as median and 95% confidence interval of the median (95% CI). Baseline values are the mean of the values in weeks five and six. The 95% CI was calculated according to the Binomial distribution with probability $1/2$.¹⁶

Statistical analysis was performed by *t*-test (paired when appropriate) or one way analysis of variance (ANOVA) with a post-test according to Bonferroni in case of normal distribution of data, otherwise by a Mann-Whitney Rank Sum Test (two groups) or a Kruskal-Wallis ANOVA on ranks with a post-test according to Dunn's method with the untreated adriamycin nephrotic rats as control group. Correlations were determined by the Pearson method. Statistical significance was assumed at the 5% level. Statistical analysis was performed by SPSS version 8.0.

Results

Protocol A: time-course of renal ACE activity in adriamycin nephrosis

At the end of this experiment, proteinuria was 877 (483-1,416) mg/day in the adriamycin nephrotic rats and 53 (26-128) mg/day in the healthy

Table 2 Expression of renal ACE mRNA and ACE activity at the end of protocol B

	ACE mRNA (ratio vs. GAPDH)	Renal ACE (nmol/g/min)	Plasma ACE (nmol/ml/min)
Untreated	0.97 (0.67-1.24)	271 (136-306)	56 (38-61)
ACE inhibitor	1.33 (1.15-1.52)*	27 (10-58)*	7 (3-9)*
ARB	1.09 (0.83-1.44)	177 (118-406)	83 (74-92)*

Median and 95% confidence interval of the median.
Renal ACE = renal ACE activity; ACE mRNA = expression of the mRNA of the ACE-gene in renal tissue homogenates; ARB = AT₁-receptor blocker; * p<0.05 versus untreated animals

control rats (p<0.001). In the adriamycin nephrotic rats, the focal glomerulosclerosis score was 20 (0-30) at week six and 107 (10-162) at week 12 (p<0.01). The control rats did not develop focal glomerulosclerosis (4 [0-16] at week 12). In the adriamycin nephrotic rats, renal ACE activity increased from 53 (12-162) nmol/g/minute at week six to 159 (20-314) nmol/g/minute at week 12 (p<0.05). In the control rats, renal ACE activity was 78 (43-101) nmol/g/minute at week 12 (p=0.181 versus adriamycin nephrotic rats at week 12).

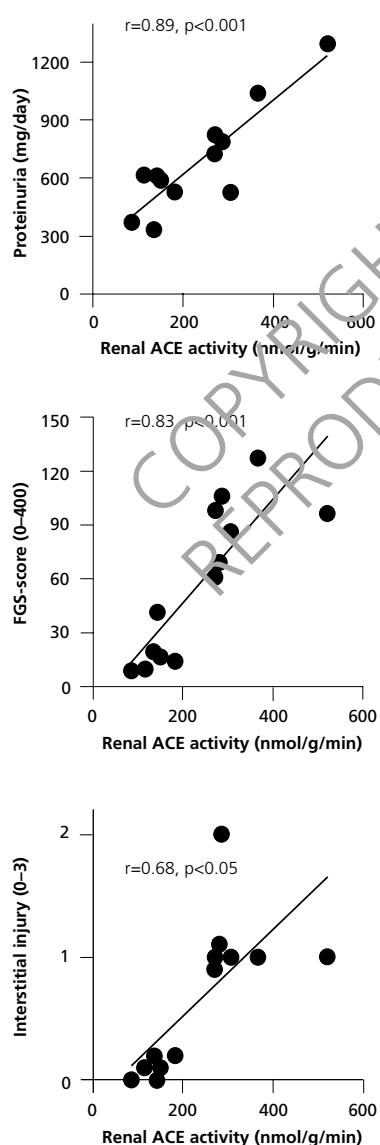
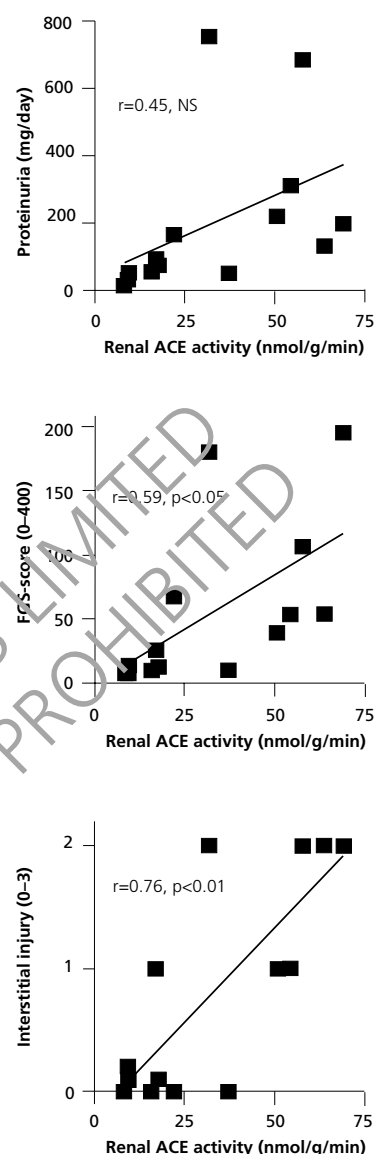
Protocol B: Renal ACE activity and renal outcome in untreated and treated adriamycin nephrotic rats

Six weeks after injection of adriamycin, proteinuria had stabilised at a level of 741 (563-836) mg/day. At stratification, baseline proteinuria and SBP were comparable in all groups (data not shown). Both the ACE-I and the ARB resulted in a reduction of BP, proteinuria, focal glomerulosclerosis and interstitial injury scores, compared with the untreated group, without any differences between the two active treatment groups (Table 1). As anticipated, residual proteinuria at the end of the study correlated with focal glomerulosclerosis and interstitial injury in all groups. Moreover, focal glomerulosclerosis correlated with interstitial injury (data not shown).

Table 3 Correlations between renal ACE activity and renal outcome at the end of the study correlation-coefficients at the end of protocol B

	Untreated	ACE-inhibitor	ARB
ACE mRNA	0.71**	0.60*	0.90***
FGS-score	0.83***	0.59*	0.71**
Interstitial injury-score	0.68*	0.76**	0.60*
Ratio of FGS to proteinuria	0.59*	0.27	0.17
Ratio of interstitial injury to proteinuria	0.56*	0.60*	0.48

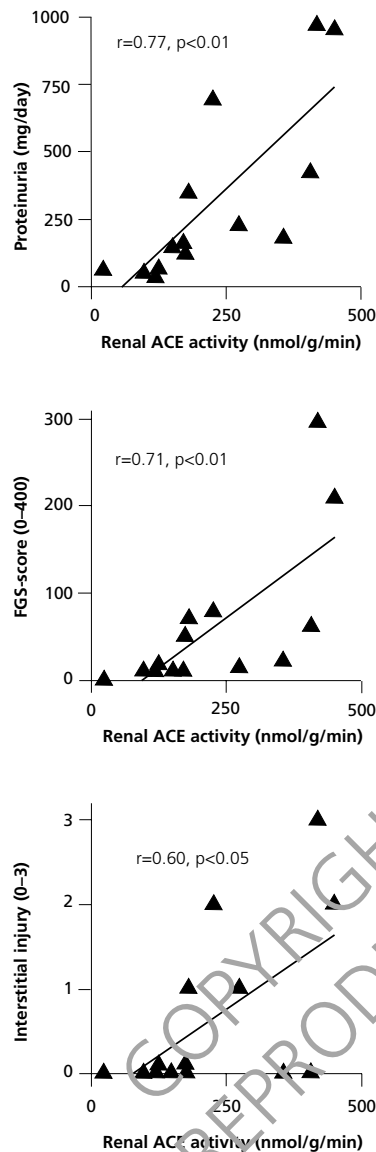
Renal ACE = renal ACE activity; ACE mRNA = expression of the mRNA of the ACE-gene in renal tissue homogenates; FGS-score and FGS = focal glomerulosclerosis-score; ARB = AT₁-receptor blocker; * p<0.05; ** p<0.01; *** p<0.001

Figure 1 Untreated adriamycin nephrotic rats. Correlation between renal ACE activity and renal outcome, expressed as proteinuria, focal glomerulosclerosis-score (FGS-score) and interstitial injury-score**Figure 2** ACE-inhibitor-treated adriamycin nephrotic rats. Correlation between renal ACE activity and renal outcome, expressed as proteinuria, focal glomerulosclerosis-score (FGS-score) and interstitial injury-score

During ACE-inhibition, but not during ARB treatment, renal as well as plasma ACE activity were significantly reduced and ACE mRNA was upregulated compared with the untreated group (Table 2). In all groups, ACE mRNA correlated with renal ACE activity (Table 3). No correlation was present between renal ACE activity and plasma ACE activity, nor between ACE mRNA and plasma ACE activity in any of the groups (data not shown).

The relationship between renal ACE activity at the end of the study and the three different renal outcome parameters (proteinuria, focal glomerulosclerosis and interstitial injury scores) is given in Figures 1, 2 and 3. In the untreated rats, renal ACE activity strongly and positively correlated with proteinuria at the end of the study ($r=0.89$, $p<0.001$), and with focal glomerulosclerosis ($r=0.83$, $p<0.001$) and interstitial injury ($r=0.68$,

Figure 3 ARB-treated adriamycin nephrotic rats. Correlation between renal ACE activity and renal outcome, expressed as proteinuria, focal glomerulosclerosis-score (FGS-score) and interstitial injury-score

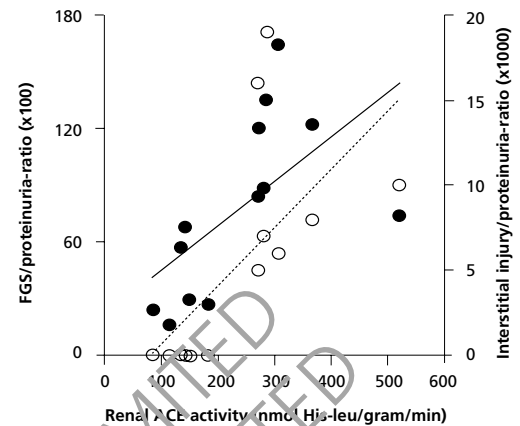


$p < 0.05$) (see Figure 1 and Table 3). In both active treatment groups, these correlations were similarly present (see Figure 2 and 3, and Table 3). No correlations were present between the renal parameters and plasma ACE activity.

If ACE activity plays a pathogenetic role in proteinuria-associated renal damage, one would expect that, for a given amount of proteinuria, higher ACE activity would be associated with more severe renal damage. This relationship is provided in Figure 4 and see Table 3, as the correlation between renal ACE activity and the ratio of focal glomerulosclerosis to proteinuria, i.e. the severity of focal glomerulosclerosis for a given proteinuria, in untreated adriamycin nephrotic rats. It shows a significant positive correlation ($r = 0.59$, $p < 0.05$). A similar correlation was found for the relationship between renal ACE activity and the ratio of inter-

Figure 4 Untreated adriamycin nephrotic rats.

Correlation between renal ACE activity and ratio of focal glomerulosclerosis-score (FGS) to proteinuria at end of study and between renal ACE activity and ratio of interstitial injury-score to proteinuria at end of study. Closed circles and continuous regression line: ratio of focal glomerulosclerosis-score to proteinuria; open circles and broken line: ratio of interstitial injury-score to proteinuria



stitial injury to proteinuria (see Figure 4 and Table 3, $r = 0.56$, $p < 0.05$). Thus, rats with higher renal ACE activity have more severe glomerular and interstitial damage for a given severity of proteinuria. These associations were not present in the groups treated with either ACE-I or ARB.

Discussion

The main finding of this study is the strong and positive correlation between renal ACE activity and proteinuria, focal glomerulosclerosis and interstitial injury in established adriamycin nephrosis. This correlation is present not only in untreated rats, but notably also during renoprotective intervention with ACE-I or ARB. The development of renal structural damage over time was paralleled by a rise in renal ACE activity. These findings suggest that renal ACE activity plays a role in proteinuria-induced renal damage, but do not exclude the possibility that renal ACE activity is merely a marker of renal damage. Moreover, we found that higher renal ACE activity is associated with more severe glomerular and interstitial damage for a given amount of proteinuria, suggesting that renal ACE activity is either a permissive, or a promoting factor in the processes by which proteinuria eventually leads to renal structural damage. This relationship was abolished by both ACE-I and ARB, suggesting that renin-angiotensin system (RAS) activity is involved in the relationship between renal ACE activity and the development of proteinuria-induced renal structural damage.

In accordance with a previous study,¹⁷ in our time-course experiment the development of renal structural damage in adriamycin nephrotic rats was paralleled by a significant rise in renal ACE activity. Moreover, on cross-sectional analysis, a close correlation was found between the three

different renal outcome parameters and renal, but not plasma ACE activity. Thus, renal ACE activity is closely associated with renal damage in this model. Remarkably, this correlation was not only present in untreated rats, but also during antiproteinuric treatment. As to the ACE-I group, it could be argued that a poor pharmacological efficacy could lead to high renal ACE activity as well as a poor renal outcome. However, the correlation was also present for the ARB, which indicates that the relationship between renal ACE activity, proteinuria and renal structural damage during antiproteinuric treatment is not specific for ACE inhibition. Thus, renal ACE activity appears to be a consistent marker of renal structural damage in this model.

Remarkably, the relationship between ACE activity and renal damage was present in a condition with low absolute levels of renal ACE activity (i.e. during ACE inhibition), as well as in conditions with high levels of renal ACE activity (i.e. during the natural course and during ARB treatment). Could this consistent association indicate a pathogenetic role of renal ACE activity in proteinuria-induced renal damage? If so, one would expect that, for a specific treatment regimen, a high renal ACE activity would be associated with a higher likelihood of developing renal structural damage during proteinuria. In accordance with the well-known features of our model,¹⁸ focal glomerulosclerosis and interstitial injury closely correlated with proteinuria at the end of the study. Thus, not surprisingly, the correlation with renal ACE activity was more or less similar for proteinuria, focal glomerulosclerosis and interstitial injury alike. To dissect the role of renal ACE activity in this interdependency, we analysed the relationship between the ratio of focal glomerulosclerosis (and interstitial injury, respectively) to proteinuria, and renal ACE activity. Remarkably, within all three groups, for any given level of proteinuria, higher renal ACE activity was associated with more severe focal glomerulosclerosis and interstitial injury, respectively. This is consistent with the hypothesis that renal ACE activity plays either a pathogenetic, or a permissive role in the mechanisms of proteinuria-induced renal structural damage. As opposed to the untreated animals, the correlation between focal glomerulosclerosis-to-proteinuria ratio (and interstitial injury-to-proteinuria ratio, respectively) was no longer present after treatment with the ACE-I or ARB, which is what one would expect if renal ACE activity affects proteinuria-induced renal damage by enhanced generation of angiotensin II (Ang II). However, it should be mentioned that our data provide no conclusive proof for a pathogenetic role of ACE activity, as we cannot exclude the possibility that this parameter is just a marker of renal damage, since we only measured ACE activity at the end of the study.

In line with our data, and those of Venkatesan,¹⁷ in the adriamycin model, Largo *et al.* observed an increase in renal ACE activity in the protein-overload model.⁸ Also, in human renal disease neo-expression of ACE by renal endothelial cells, as

well as changes of the tubular ACE content, is described in diseased kidneys.¹⁹

The experiments in the protein-overload model showed that the elevated ACE-expression was mainly localised in proximal and distal tubules and in the glomeruli.⁸ The authors hypothesise that the elevated ACE activity enhances the generation of Ang II, which could subsequently lead to renal structural damage by playing a role in tubulo-interstitial inflammation. This would fit in well with our findings. Our data extend the relationship between renal ACE and proteinuria also to the treated condition and, moreover, show that this relationship is relevant to outcome for individual rats.

By what mechanism could renal ACE activity be involved in the pathogenesis of renal structural damage? Generation of Ang II might be involved. However, it is assumed that the hydrolysis of angiotensin I by ACE is not rate-limiting in the generation of Ang II. Yet it has not been excluded that, under certain circumstances and in certain compartments of the kidney, ACE is rate-limiting. Moreover, the ACE has a wide range of other substrates, including bradykinin, and stimulates prostaglandin release and the nitric oxide pathway.²⁰ These properties of ACE could also contribute to the progression of renal structural damage.

Gene transfection experiments have provided evidence that a high ACE activity can have functional effects. After *in vivo* gene transfection of human ACE enzyme vector into intact rat carotid arteries, resulting in an increase of ACE activity, an increase in the wall-to-lumen ratio was observed in these arteries compared with control arteries, which could be prevented by the ARB, losartan.²¹ These findings suggest that the induced increase in ACE activity results in an increase in the generation of Ang II.

In conclusion, during the natural course of adriamycin nephrosis, a significant rise in renal ACE activity was observed. In untreated, as well as in treated adriamycin nephrotic rats, renal ACE activity is associated with proteinuria. Moreover, both in untreated rats and in rats treated with RAS blockade, a higher renal ACE activity is associated with more severe renal structural damage. This appears to be independent of the specific mode of blockade of the RAS. Renal ACE activity is a consistent marker of individual differences in proteinuria-associated renal damage: further studies are needed to investigate a possible pathogenetic role in renal damage.

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