INDIVIDUAL DIFFERENCES IN RENAL ACE ACTIVITY IN HEALTHY RATS PREDICT SUSCEPTIBILITY TO ADRIAMYCIN INDUCED RENAL DAMAGE

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Abstract

In man, differences in ACE levels, related to ACE (I/D) genotype, are associated with renal prognosis. This raises the hypothesis that individual differences in renal ACE activity are involved in renal susceptibility to inflicted damage. Therefore, we studied the predictive effect of renal ACE activity for the severity of renal damage induced by a single injection of adriamycin in rats.

Renal ACE activity (Hip-His-Leu-cleavage by cortical homogenates) was determined by renal biopsy in 27 adult male Wistar rats. After one week of recovery, proteinuria was induced by adriamycin (1.5 mg/kg i.v. n=18; controls: saline i.v. n=9). Proteinuria was measured 2-weekly. After 12 weeks, rats were sacrificed and kidneys harvested.

As anticipated, adriamycin elicited nephrotic range proteinuria, renal interstitial damage and mild focal glomerulosclerosis. Baseline renal ACE positively correlated with relative rise in proteinuria after adriamycin (r=0.62, p<0.01), renal interstitial α-smooth muscle actin (r=0.49, p<0.05), interstitial macrophage influx (r=0.56, p<0.05), interstitial collagen III (r=0.53, p<0.05), glomerular α-smooth muscle actin (r=0.74, p<0.01) and glomerular desmin (r=0.48, p<0.05). Baseline renal ACE did not correlate with focal glomerulosclerosis (r=0.22, ns). In controls, no predictive values for renal parameters were observed.

Individual differences in renal ACE activity predict the severity of adriamycin-induced renal damage in this outbred rat strain. This supports the assumption that differences in renal ACE activity predispose to a less favorable course of renal damage.
**Introduction**

Tissue angiotensin converting enzyme (ACE) is involved in cardiovascular and renal tissue damage [1]. ACE inhibition reduces proteinuria and protects against progressive renal damage [2;3]. This suggests that renal ACE activity might play a pathogenic role in the development of proteinuria-associated structural renal damage.

In man, the I/D polymorphism of the ACE gene accounts for half of the variance of circulating and tissue ACE levels, with the highest ACE levels in DD homozygotes, lowest in II homozygotes, and in-between values in heterozygotes [4;5]. The D-allele of the ACE genotype was reported to be associated with worse renal prognosis in several renal conditions [6], albeit not uniformly so [7;8]. The initiation of renal disease, however, does not appear to be associated with the D-allele. Based on these data, it has been hypothesized that genetically determined individual differences in renal ACE activity are relevant to the extent of renal damage that develops in response to injury [9], and more specifically, that a higher renal ACE activity predisposes to a more progressive course of renal damage. In line with this assumption, established renal damage was associated with elevated renal ACE activity in different rat models of renal disease [10;11]. These data, however, were invariably obtained after development of renal damage. Therefore they do not allow to identify renal ACE as a factor predisposing to renal damage, as the elevated ACE activity might just as well have been the consequence of renal damage.

To test, therefore, whether higher renal ACE activity predisposes to a more progressive course of renal damage, we prospectively determined renal (and plasma) ACE activity in healthy Wistar rats prior to induction of nephrosis by a single injection of adriamycin, and investigated its predictive value for the subsequent development of renal damage. Adriamycin nephrosis provides a well-characterized model of progressive renal damage, induced by a uniform challenge at a single point in time. This results in proteinuria and subsequent structural renal damage with a relative large variability between individual animals. In healthy out-bred Wistar rats, the plasma and tissue ACE levels display a relatively large inter-individual variability, which allows to test for the predictive value of naturally occurring differences in ACE activity.

**Methods**

**General**

Thirty male Wistar rats (Hsd.Cpb.Wu; Harlan Inc, Horst, The Netherlands) with an initial weight of 323 (314-332) g (median and 95% confidence interval) were studied. The animals were housed in a temperature-controlled room with a 12h light/dark cycle and had free access to standard food and tap water. Twenty-four hour urine samples were collected and food and water intake were measured by means of metabolic cages, at baseline (prior to renal biopsy), and at two week intervals throughout the study. One animal died before renal biopsy and one animal the day after. During the course of the
experiment, one more animal died at week 8. The protocol, as described below, 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.

Protocols

After baseline values had been obtained, renal biopsy was performed under isoflurane/O2/N2 anesthesia. Tissue was resected from the lower pole of the right kidney through dorsolateral incision. Local hemostasis was obtained by application of gelfoam (Spongostan®, Ferrosan, Copenhagen, Denmark). The median amount of renal tissue removed was 0.33 (0.29-0.39) g. Renal tissue was further processed for determination of ACE activity as described below. During the same procedure, blood-samples were obtained by orbital puncture for determination of plasma ACE activity and creatinine.

After one week of recovery from biopsy, nephrosis was induced by a single i.v. injection of adriamycin under light anesthesia. Nineteen rats received 1.5 mg/kg adriamycin by tail vein. Nine rats serving as controls received saline.

Twelve weeks after the induction of nephrosis, animals were anesthetized and blood pressure was measured. Subsequently, the abdomen was opened through midline incision. A two-ml blood sample

| Table 1 | Group characteristics at baseline and termination at week 12 |
|---------|-----------------|-----------------|
|         | Adriamycin (n=18) | Control (n=9) |
| Body weight (g) | Baseline 320 (318-328) | 320 (314-334) |
|           | Week 12 461 (448-480) | 490 (458-524) |
| Biopsy tissue weight (g) | Left 0.33 (0.29-0.40) | 0.30 (0.19-0.42) |
| Kidney weight at termination (g) | Right 2.23 (1.97-2.41) | 1.92 (1.65-2.29) |
| Renal ACE (nmol HL/mL/min) | Baseline 56.0 (45.1-70.6) | 54.7 (38.7-66.4) |
|           | Week 12 47.2 (34.7-59.9) | 48.2 (33.9-80.9) |
| Plasma ACE (nmol HL/mL/min) | Baseline 24.7 (21.5-29.2) | 29.9 (22.9-34.7) |
|           | Week 12 48.3 (44.4-56.2) | 52.1 (39.2-65.4) |
| Plasma creatinine (mg/L) | Baseline 5.1 (4.9-5.5) | 5.9 (2.5-6.5) |
|           | Week 12 6.0 (5.6-6.3) | 4.9 (4.8-5.3) |
| Proteinuria (mg/24h) | Baseline 31 (17-41) | 28 (18-45) |
|           | Week 12 268 (148-347) | 26 (14-123) |

Data are expressed as median and 95% confidence interval of the median, a p<0.05 adriamycin versus control, b p<0.01 adriamycin versus control, c p<0.01 termination (week 12) versus baseline, d p=0.01 termination (week 12) versus baseline
was obtained via aortic puncture for determination of plasma ACE activity and creatinine. Kidneys were saline perfused and harvested and animals were sacrificed. Renal cortical tissue from the upper pole was processed for ACE determination. Mid-coronal renal tissue slices were processed for histological and immunohistochemical examination.

**Measurements**

Urinary protein excretion was measured by means of a third generation nephelometer (Dade Behring, Mannheim, Germany) by using a 20% trichloroacetic acid (TCA) solution. Systolic blood pressure (SBP) was measured by tail cuff. Plasma creatinine level was determined colorimetrically (Sigma Chemical Co, St Louis, MO, USA).

ACE activity was determined as described previously [10]. Renal cortex tissue was homogenized in a 50 mM K2PO4 buffer at pH 7.5. Subsequently, 100 µl of the diluted sample was pipetted into a 0.5 M K2PO4 buffer. Then, the substrate (100 µl of 12.5 mM Hip-His-Leu (Sigma)) which is cleaved by ACE, was added. This was incubated at 37°C for exactly 15 minutes. In this amount the substrate is present in excess, and thus not rate-limiting for the reaction. The conversion of the substrate was stopped by adding 1.45 ml of 280 mM sodiumhydroxide. Then, 100 µl of 1% phtaldialdehyde, which adheres to the formed bipeptid His-Leu, was added. The amount of tagged His-Leu was fluorimetrically determined at 364 nm excitation wavelength and 486 nm emission wavelength. This yields a measure of the amount of His-Leu generated in the sample. In blank samples, sodiumhydroxide was added to prevent conversion. The substrate was added after the incubation period. The coefficient of variation was 6% for these measurements of ACE activity using this method.

<table>
<thead>
<tr>
<th></th>
<th>Adriamycin (n=18)</th>
<th>Control (n=9)</th>
<th>p-value a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomerular mø (per 50 glomeruli)</td>
<td>79 (51-101)</td>
<td>52 (45-71)</td>
<td>0.08</td>
</tr>
<tr>
<td>Glomerular α-SMA (mean % of 50 glomeruli)</td>
<td>3.3 (2.0-4.5)</td>
<td>1.8 (1.4-2.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Glomerular desmin (mean % of 50 glomeruli)</td>
<td>20 (10-32)</td>
<td>1.6 (0.3-17)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MME score (per 50 glomeruli, scale 0-200)</td>
<td>42 (10-32)</td>
<td>27 (20-44)</td>
<td>0.09</td>
</tr>
<tr>
<td>FGS score (per 50 glomeruli, scale 0-200)</td>
<td>19 (6-34)</td>
<td>0 (0-8)</td>
<td>0.01</td>
</tr>
<tr>
<td>Interstitial mø (per 30 cortical fields)</td>
<td>94 (66-119)</td>
<td>30 (23-42)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Interstitial α-SMA (mean % of 30 fields)</td>
<td>4.2 (3.4-6.8)</td>
<td>2.6 (1.4-4.1)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Interstitial collagen III (mean % of 30 fields)</td>
<td>16.6 (13.5-17.3)</td>
<td>10.5 (8.6-14.9)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Data are expressed as median and 95% confidence interval of the median. mø: macrophage count; α-SMA: α-smooth muscle actin; MME: mesangial matrix expansion, FGS: focal glomerular sclerosis a adriamycin versus control: Mann-Whitney U test
**Histological procedures**

Renal tissue was fixed in 4% paraformaldehyde and processed for paraffin embedding. For morphological evaluation, 4 μm sections were cut. One series was stained with periodic acid-Schiff (PAS). Another series was stained with polyclonal rabbit anti-rat collagen III antibody (Biogenesins Ltd, Poole, UK). An automated staining system was used on series for macrophages (ED-1, Serotec Ltd, Oxford, UK), the pre-fibrotic markers alpha-smooth muscle actin (α-SMA; clone 1A4; Sigma Aldrich, St Louis, MO, USA) and desmin (clone D33, DAKO Cytomation, Glostrup, Denmark). Sections were first dewaxed and subjected to heat induced antigen retrieval by overnight incubation in 0.1 M Tris/HCl buffer at 80°C. Endogenous peroxidase was blocked with 0.075% H2O2 in phosphate-buffered saline (PBS) for 30 minutes. Antibody dilutions were made in PBS supplemented with 1% bovine serum albumin. Antibody binding was detected using sequential incubations with peroxidase-labeled rabbit anti-mouse and peroxidase-labeled goat anti-rabbit antibodies (RAMPO/GARPO Dakopatts, DAKO). Normal rat serum (1%) was added to the secondary antibodies. Peroxidase activity developed by using 3,3-diaminobenzidine tetrachloride for 10 minutes.

PAS stained sections were used for determination of focal glomerular sclerosis (FGS) and mesangial matrix expansion (MME). The degree of FGS and MME were assessed by scoring 50 glomeruli per kidney semiquantitatively on a scale of 0 to 4. FGS was scored positive when mesangial matrix expansion and adhesion of the glomerular visceral epithelium to Bowman’s capsule were present in the same segment. If 25% of the glomerulus was affected, a score of 1 was adjudged, 50% was scored as 2, 75% as 3 and 100% as 4. The ultimate score is obtained by multiplying the degree of change by the percentage of glomeruli with the same degree of injury and adding these scores, thus rendering a theoretical range of 0 to 200.

The number of glomerular ED-1 positive cells was determined by manual counting of 50 glomeruli per kidney. Interstitial macrophage number was determined by manual counting of 30 cortical fields per kidney.

Interstitial α-SMA and collagen III were measured using computer-assisted morphometry on 30 cortical fields per kidney, excluding vessels and glomeruli. The surface area found positive, was divided by the total area of the field measured, providing a percentage of α-SMA positive tissue. Glomerular α-SMA was determined on 50 glomeruli per kidney. The glomerular visceral epithelial desmin staining was determined by manual counting of 50 glomeruli per kidney, on a scale of 0 to 100%, with intervals of 10%.

**Statistical Analysis**

Statistical analyses were performed on a total of 27 animals: 18 adriamycin animals and 9 controls. The analysis on predictive value of endogenous renal ACE activity was performed on data obtained from
the right kidney at biopsy and at termination. Left kidneys obtained at termination were compared to the right kidney termination data to test for possible harmful consequences of the biopsy.

Data are expressed as median and 95% confidence interval of the median, calculated according to the ranks method by Altman. Differences between experimental and control groups were analyzed with Mann-Whitney U test. Wilcoxon’s non-parametric test for paired samples was used to compare parameters from baseline to termination. Pearson’s coefficients were calculated to account for bivariate correlation. Statistical significance was assumed at the 5% level. Analyses were performed by SPSS version 10.0.

Results

Clinico-pathological parameters

Values for clinical parameters at baseline and termination are listed in table 1. Food and water intake was similar in both groups throughout the experiment. Body weight was significantly lower in the adriamycin rats as compared to controls, from week 8 onwards (p<0.05, data not shown) until termination at week 12 (p<0.001). Median systolic blood pressure at baseline was 88 mmHg (77-95) for the two groups together, without differences between the groups. At week 12, blood pressure was slightly higher in the adriamycin rats as compared to controls (134 (125-141) versus 124 (113-130) mmHg, ns). Subtle proteinuria with a median value of 31 (18-40) mg/24h was present at baseline. In the adriamycin group, proteinuria showed a marked rise after adriamycin induction to overtly proteinuric values (268 (148-347) versus 26 (14-123) mg/day in controls at week 12 (p<0.001). Plasma creatinine was similar in both groups at baseline. At termination, plasma creatinine was significantly higher in the adriamycin group (p<0.01). Wet kidney weight at termination was similar in both groups.

![Figure 1](image.png)

Figure 1 | The predictive value of baseline renal ACE activity for the % change in adriamycin induced proteinuria
Structural changes and markers of renal damage

Data on structural renal damage at termination are shown in table 2. For mesangial matrix expansion (MME) we found somewhat higher values in adriamycin animals, but this difference did not reach statistical significance. In the adriamycin group, mild focal glomerulosclerosis (FGS) was found. Macrophage-influx was significantly elevated in the adriamycin group as compared to control, both for interstitial and glomerular macrophages. Both interstitial and glomerular \( \alpha \)-SMA expression and glomerular epithelial desmin expression were significantly increased in the adriamycin group. Interstitial collagen III expression was significantly elevated in adriamycin animals. All immunohistological parameters at termination were similar for left and right kidney in both groups, indicating that these parameters were not affected by the prior renal biopsy (data not shown).

Predictive value of baseline parameters for adriamycin-induced renal damage

Baseline renal ACE activity significantly correlated with the rise in proteinuria induced by adriamycin (figure 1). Bivariate correlations between baseline renal ACE activity and renal damage at termination in the adriamycin rats are given in figure 2. It shows that baseline renal ACE activity correlated positively and significantly with the expression of glomerular markers \( \alpha \)-SMA and desmin and with interstitial \( \alpha \)-SMA and collagen III expression in adriamycin rats. Renal ACE activity at
baseline predicted interstitial macrophage influx at termination as well (r=0.56; p<0.05). Glomerular macrophage count (r=0.13; p=0.62), MME and FGS did not correlate with baseline renal ACE activity (r=0.33; p=0.18 and r=0.22; p=0.37, respectively). Plasma ACE values at baseline had no predictive effect for any of the renal parameters. In control animals, neither renal nor plasma ACE had a predictive effect for any of the renal parameters.

On cross-sectional analysis of termination data in adriamycin animals, renal ACE activity at termination significantly correlated with proteinuria (r=0.68; p<0.01), interstitial macrophages (r=0.57; p=0.01), interstitial α-SMA (r=0.65; p<0.01), glomerular desmin (r=0.72; p<0.01), glomerular α-SMA (r=0.75; p<0.01) and FGS (r=0.61; p<0.01) but again not with glomerular macrophage influx (r=0.07; p=0.77). For collagen III and MME the correlations were of borderline significance (r=0.47; p=0.05 and r=0.49; p=0.06, respectively). All parameters for renal damage at termination correlated with week 12 proteinuria (data not shown).

Finally, the between-individual differences in renal and plasma ACE activity were consistent between the start and the end of the study. This is illustrated in figure 3, providing the correlation between renal ACE at baseline and at termination (upper panel) and the correlation between plasma ACE at baseline and at termination (lower panel, n=16 for adriamycin and n=8 for control animals).

**Discussion**

Individual differences in renal, but not plasma, ACE activity predicted the rise in proteinuria induced by adriamycin, the severity of renal interstitial changes and the expression of the glomerular markers α-SMA and desmin. This supports the hypothesis that individual differences in renal ACE level are relevant to the progression of renal damage.

The differences in baseline renal ACE activity can be considered to reflect naturally occurring differences between these outbred animals. Interestingly, in these adriamycin rats, the between-

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**Figure 3** | The correlation between baseline ACE activity with termination ACE activity for adriamycin and control animals

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ACE activity and renal damage
individual differences found at baseline were consistent throughout the study from the pre-induction values in normal kidney through the termination values in diseased kidney. This consistency strongly suggests that the values reflect a true difference between the animals rather than a random fluctuation at the time of biopsy.

It could be argued that the renal biopsy affected the renal outcome in our study. However, no morphological or immunohistochemical differences between the biopsied and the non-biopsied kidney were found at termination. Renal ACE activity at termination was similar in biopsied kidney compared to non-biopsied kidney. Thus, whereas effects of the renal biopsy cannot be excluded, they do not modify the conclusions of our study.

In the time-controls, median proteinuria was low and stable throughout the study, and values at termination were not predicted by baseline ACE. Considering the absence of substantial abnormalities at termination in these animals, the likelihood to detect a predictive effect within the timeframe of the study was low. It would be of interest, therefore, to study long-term age-related changes in this strain in relation to baseline renal ACE activity.

Baseline renal ACE predicted the severity of interstitial and glomerular prefibrotic changes— but not focal glomerular sclerosis and MME. Within the timeframe of our study the glomerular sclerotic changes, however, remained mild. Accordingly, the quantitative resolution to detect a relationship with the glomerular sclerotic changes may have been too limited. The predictive value of renal ACE for expression of the prefibrotic glomerular markers suggests, that a consistent dissociation between interstitial and glomerular changes is unlikely. In this model interstitial morphological changes have been noted to prevail over glomerular changes [11]. Nevertheless, during advanced stages of renal damage in this model, usually interstitial and glomerular changes go hand in hand [12]. Therefore, it would be worthwhile to investigate whether during long-term follow-up, baseline ACE predicts the progression of glomerular sclerosis as well.

Our data show the predictive value of baseline ACE for renal damage but do not provide proof for a causal role of renal ACE activity in the differences in renal damage, as we cannot exclude that it is an epiphenomenon to - unknown – associated factors. Study designs with intervention in renal ACE activity, either pharmacological or genetic, would be needed for conclusive proof. The renoprotective effect of ACE-inhibition in this model, as well as other proteinuric models [2;13-17] is well-established, and in line with a role of renal ACE in renal damage, albeit not conclusively, as the relationship between baseline renal ACE and pharmacodynamics of ACE-inhibition is complicated, and has not been well-characterized.

Recent data in mice with extra copies of the ACE gene provide specific data on this issue. These genetically engineered mice have modestly elevated plasma ACE and kidney ACE mRNA, but do not
develop spontaneous renal damage. After induction of diabetes they develop more severe hypertension and renal damage than animals with one or two copies [18]. Thus, a genetically elevated ACE activity does not elicit renal damage, but when damage is inflicted; the course is more severe, which is well in line with our data in outbred rats.

By what mechanism could renal ACE activity modify the course of renal damage? Egido’s group [19] postulated that elevated ACE activity in tubular and interstitial cells leads to generation of angiotensin II, which contributes to tubulo-interstitial damage by its pro-inflammatory and pre-fibrotic effects. In experimental renal disease (protein-overload) and human diabetic nephropathy [20], these authors found higher renal ACE activity when renal damage was present. This is in line with a prior study from our group in adriamycin nephrosis [21]. These data were obtained after development of renal damage, however. The higher renal ACE in association with more severe damage might be the result from renal damage, rather than a predisposing factor. Our present data show that individual renal ACE levels in the healthy condition precede, and thus predict the course of renal damage. Moreover, the predictive value was observed despite stable renal ACE levels throughout the study – showing that the predictive effect of baseline renal ACE does not depend on a disease-associated rise in renal ACE level. The absence of a rise in renal ACE activity in our study is somewhat at variance with increased renal ACE activity in some other studies [19]. This may be due to differences between the models, or to the relatively mild extent of damage in our study.

In conclusion, our data show that naturally occurring individual differences in renal ACE levels predict the susceptibility to adriamycin-induced renal injury. This suggests that differences in renal ACE activity predispose to a more aggressive course of renal damage. Future studies are needed to investigate the underlying mechanisms, and to explore whether this predictive effect also applies to other models of renal damage and in human renal disease.

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References


