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Therapeutic resistance to angiotensin converting enzyme (ACE) inhibition is related to pharmacodynamic and –kinetic factors in 5/6 nephrectomized rats

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Dick de Zeeuw
Robert H. Henning
**ABSTRACT**

**INTRODUCTION** Proteinuria plays a pathogenic role in the development of end stage renal disease. Angiotensin converting enzyme inhibitors (ACEi) lower proteinuria and are renoprotective. However, large inter-individual variation in antiproteinuric response to ACEi exists. In this study, we explored the mechanism behind this therapy resistance to ACEi in the rat 5/6 nephrectomy model.

**METHODS** At week 6 after 5/6 nephrectomy, treatment with lisinopril was initiated for 6 weeks. Proteinuria and blood pressure were evaluated weekly. At the end of the experiment, rats were divided into tertiles according to their anti-proteinuric response: (1) responders (RT, n=9), (2) intermediate responders (IRT, n=8) and (3) non-responders to ACE-I therapy (NRT, n=9).

**RESULTS** At start of treatment, proteinuria had progressively increased to 154 (95% confidence interval (CI): 123 to 185) mg/24h in the entire cohort, with comparable proteinuria and blood pressure in all groups. Following treatment with ACEi, proteinuria was significantly lower in the RT-group (68 (95%CI 46 to 89) mg/24h) compared to the NRT-group (251 (95%CI 83 to 420) mg/24h). Similarly, blood pressure was reduced in RT, but unaffected in NRT. At autopsy, renal ACE activity and renal ACE expression were significantly lower in RT compared to NRT. Although lisinopril intake was comparable in all animals, urinary drug excretion was increased in NRT animals, demonstrating increased drug clearance. Average urinary lisinopril excretion was correlated with anti-proteinuric response ($R^2= 0.32$, $P= 0.003$).

**CONCLUSION** Both pharmacodynamic and –kinetic factors account for the non-response to lisinopril. Whether these can be overcome simply by increasing drug dosage in non-responders should be investigated.
INTRODUCTION
Angiotensin converting enzyme inhibitors (ACEi) improve renal outcome in renal disease, in humans as well as in the experimental setting. Both the reduction in proteinuria and blood pressure appear to play a role in the prevention of structural renal damage. However, not every patient with chronic renal failure benefits from ACEi optimally, due to the large inter-individual variation in therapy response to these drugs, which seems only marginally modulated by changing dose within the recommended range or the class of drug. Because the reduction in proteinuria is correlated to renal prognosis, it is of the utmost importance to reduce proteinuria to the lowest possible level. Insight in the mechanisms of the resistance to the anti-proteinuric efficacy of ACEi may provide clues for optimizing therapy and renal prognosis in patients with chronic renal failure. Different mechanisms underlying therapy resistance to ACEi have been identified both in clinical and experimental settings. Therapy response to ACEi has been shown dependent on sodium status, ACE gene polymorphism, activation of the renin angiotensin aldosterone system (RAAS), and the extent of renal damage prior to ACEi therapy. It is however unclear to what extent these factors alter pharmacodynamic or -kinetic properties of ACEi. In previous experiments, we found a large variation in therapeutic response to lisinopril in 5/6 nephrectomized rats. As in untreated animals, renal ACE expression predicts the progression of renal disease and in humans renal ACE is up regulated in renal disease, we hypothesized a critical role for renal ACE activity in therapy resistance to ACEi. To test this hypothesis, we compared renal ACE expression and ACE activity in responders and non-responders to lisinopril treatment in 5/6 nephrectomized (5/6NX) rats. With regard to possible differences in the pharmacokinetic profile explaining therapeutic resistance to ACEi, we investigated drug concentrations and excretion. Renal ablation by 5/6NX is a hypertensive model provoking both renal and cardiac damage. While ACEi exerts beneficial effects on both the heart and the kidney, its effects on cardiac parameters were measured as well to investigate variation in cardiac therapy response.

MATERIALS AND METHODS
Experimental Protocol
Male Wistar rats (275-350 g; n=33) were housed under standard conditions with free access to food and drinking water. Rats received a standard chow diet. Animal experiments were approved by the institutional animal ethical committee. One rat died within 24 hours after the surgical procedure. At initiation of the experiment (t= 0 wks), animals underwent 5/6 nephrectomy (5/6NX). After 6 weeks, the animals were randomized based on their proteinuria prior to drug treatment into a group subsequently treated with lisinopril (Merck Sharp & Dohme, Haarlem, The Netherlands) 2.5 mg/kg/day in the drinking water (ACEi, n=26), and a vehicle treated group (VEH, n=7). In both groups, the experiment was terminated 12 weeks after baseline. To standardize drug intake in individual animals at the end of the experiment, the final dose of lisinopril (2.5 mg/kg in 500 μl ml water) was administered by gavage 24h before sacrifice. At the end of the experiment, at a through level of lisinopril, functional cardiac parameters were measured under 2.5% isoflurane anesthesia, laparotomy was performed and renal blood flow was measured, followed by exsanguination by taking blood samples from the abdominal aorta for plasma measurements. The remaining kidney was flushed with saline and the heart and kidney were removed, weighed and processed further.
Surgical interventions

5/6th Nephrectomy was performed under anesthesia with 2.0% isoflurane in N₂/O₂ (2:1) as described before. Shortly, the right kidney was removed after ligation of the renal artery, vein and ureter. In addition, the proximal branch of the left renal artery (often responsible for 2/3 of the blood supply to the kidney) was ligated upon and interruption of 2/3 of the blood supply to this kidney was determined by visual inspection. If necessary, additional (smaller) branches of the renal artery were ligated.

Functional cardiac and renal characteristics

Cardiac performance was measured with a pressure transducer catheter under anesthesia, using 2.5% isoflurane in O₂ through the right carotid artery (Micro-Tip 3French, Millar Instruments Inc., Houston, TX, USA), connected to a personal computer equipped with an analog-to-digital converter and appropriate software (Millar Instruments, Germany). After a 3-min period of stabilization, left ventricular end-diastolic pressure (LVEDP), left ventricular peak-systolic pressure (LVPESP) and heart rate were recorded. Thereafter, the catheter was withdrawn into the aortic root to measure central systolic blood pressure (SBP). As a parameter of global myocardial contractility and relaxation, we determined the maximal rates of increase and decrease in left ventricular pressure (systolic +dP/dt_{max} and diastolic -dP/dt_{min}), which were normalized to left ventricular pressure change (i.e., LVPESP-LVEDP) for individual rats. Renal blood flow was measured using a 1 mm flow probe around the left renal artery (1RB; Transonic, Ithaca, NY, USA), connected to a flow meter (T106 Small Animal Research Flow meter, Transonic, Ithaca, NY, USA).

Histology

Kidneys were fixed by immersion for 48 hours in a 4% buffered formaldehyde solution (Klinipath, Duiven, The Netherlands) after longitudinal bisection and subsequently embedded in paraffin according to standard procedures. Sections of 3 µm were stained with periodic acid Schiff (PAS). The degree of focal glomerulosclerosis (FGS) was assessed in 50 glomeruli by scoring semi-quantitatively on a scale of 0 to 4. FGS was scored positive when mesangial matrix expansion and adhesion to Bowman's capsule was present in the same quadrant. When 25% of the glomerulus was affected, a score of 1+ was adjudged, 50% was scored as 2+, 75% as 3+ and 100% as 4+. Overall FGS score is expressed as arbitrary units (AU) with a maximum of 200. An examiner blinded for the groups evaluated all sections. Interstitial alpha-smooth muscle actin (α-SMA) was determined as a profibrotic marker and detected in paraffin-embedded sections by means of a mouse monoclonal α-SMA antibody (Sigma Chemical, St. Louis, MO, USA). The antibody was incubated for 60 min and its binding detected by sequential incubations with peroxidase (PO)–labeled rabbit anti-mouse and PO-labeled goat anti-rabbit antibody (both from Dakopatts, DAKO, Glostrup, Denmark) for 30 min. The expression of interstitial α-SMA was measured by computerized morphometry. Therefore, 40 fields were scored at 20x magnification in the cortical region. Glomeruli and blood vessels were excluded from the cortical region by extracting them along Bowman's capsule and the vessel wall. Total staining was expressed as percentage of total area surface.
Clinical chemistry

For the measurement of urinary total protein, 24-hour urine samples were collected in metabolic cages every week during the experiment. Urinary total protein was analyzed using endpoint measurement with TCA (Nephelometer Analyzer II, Dade Behring, Marburg, Germany). As a measure for renal function, creatinine clearance was investigated. For the calculation of creatinine clearance, urinary creatinine and plasma creatinine levels were measured at baseline, before start of therapy and at the end of the experiment. Creatinine was determined using photometric determination with the Jaffe method (Ecoline Mega, DiaSys Diagnostic Systems GmbH, Holzheim, Deutschland). At the end of the experiment, plasma and renal ACE activity were measured to investigate the therapy efficacy of the ACE inhibitor using the conversion of hippuryl-His-Leu (Sigma) by ACE to free His-Leu [24]. For this measurement and the lisinopril measurements, renal tissue was homogenized in 50 mM KPO4 buffer. Lisinopril levels were measured in plasma and kidney homogenate at autopsy and in urine at wk 7, 10 and 12. Lisinopril and internal standards (enalaprilat for plasma and urine; ramipril for kidney homogenate) were measured by HPLC-MS/MS [25] on a Scieix 3000 triple quadrupole massspectrometer (Applied Biosystems/ MDS Scieix, Concord, Ont, Canada). The lisinopril and internal standard were extracted from rat plasma, urine and kidney homogenate by a solid-phase extraction method. Briefly, to 400μl sample, 100μl 0.1 mol/L HCL, 4mL isopropanol/ethyl acetate (1:2, v:v) was added and vortexed. The upper organic layer was evaporated to dryness at 50°C under a gentle stream of nitrogen. The dry residues were reconstituted to 400 μl with mobile phase (water and methanol 95:5 (v:v) with 0.1% HCOOH) and filtrated through a 0.2 μm pore centrifugal device (Pall Life Sciences, Ann Arbor, USA). A 20 μl aliquot of the resulting solution was injected into the HPLC/MS/MS system for analysis. Calibration curves for lisinopril were prepared by spiking blank plasma, renal tissue homogenate and urine at concentrations of 1 to 250 ng/ml for plasma and renal tissue homogenate and at concentrations of 0.5 to 15 μg/ml for urine. The analyses were performed in duplicate for each concentration. Correlation coefficients of 0.99 for plasma, 0.97 for renal tissue homogenate, and 0.99 for urine confirmed that the calibration curves were linear over the concentration range mentioned.

Calculations and statistical analysis

At the end of the experiment, rats that received ACEi were divided into 3 groups according to their anti-proteinuric response. For this, the change in proteinuria compared to randomization was calculated for each animal and plotted for each animal. Individual antiproteinuric response was defined as the area under the curve (AUC) to rule out sampling errors. The AUC was measured with Sigmaplot 9.01 (Systat Software Inc, Richmond, CA, USA). The animals were ranked from low to high AUC in (1) responders to ACEi therapy (n=9), (2) intermediate responders to ACEi therapy (n=8) and (3) non-responders to ACEi therapy (n=9).

All data are presented as mean with 95% confidence interval or mean ± SEM in figures. Statistical analysis was performed by one-way ANOVA, followed by a LSD post hoc test to identify the groups that were different from each other in case of normally distribution. If data were not normally distributed, a log transformation was performed or a Kruskal Wallis test was used, followed by a Mann-Whitney test to determine which groups differed from each other. To identify differences in one animal between two time points, a paired sample t-test was used. In case the data were not normally distributed, a Wilcoxon signed ranks test was used. Statistical significance was assumed at the 5% level.
Table 1. Renal and hemodynamic characteristics

<table>
<thead>
<tr>
<th></th>
<th>Time point</th>
<th>VEH</th>
<th>ACEi NRT</th>
<th>ACEi IRT</th>
<th>ACEi RT</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td></td>
<td>7</td>
<td>9</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>Antiproteinuric AUC</td>
<td>treatment</td>
<td>109 (-202;419)</td>
<td>128 (-68;324)</td>
<td>-148 (-203;-91)</td>
<td>-313 (-344;-282)</td>
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<tr>
<td>Total protein excretion</td>
<td>randomization</td>
<td>134 (89;180)</td>
<td>143 (63;222)</td>
<td>184 (80;287)</td>
<td>153 (108;198)</td>
</tr>
<tr>
<td></td>
<td>autopsy</td>
<td>275 (190;361)</td>
<td>251 (83;420)</td>
<td>148 (77;218)</td>
<td>68 (46;89)ab</td>
</tr>
<tr>
<td>Creatinine clearance</td>
<td>randomization</td>
<td>3.2 (2.7;3.6)</td>
<td>2.9 (2.1;3.8)</td>
<td>2.6 (2.3;3.0)</td>
<td>3.4 (2.7;4.1)</td>
</tr>
<tr>
<td></td>
<td>autopsy</td>
<td>2.4 (1.3;3.5)</td>
<td>2.5 (1.5;3.6)</td>
<td>2.4 (1.5;3.2)</td>
<td>2.3 (1.7;2.9)</td>
</tr>
<tr>
<td>Systolic Blood Pressure</td>
<td>randomization</td>
<td>158 (131;185)</td>
<td>165 (147;183)</td>
<td>190 (163;218)</td>
<td>177 (163;192)</td>
</tr>
<tr>
<td></td>
<td>autopsy</td>
<td>164 (148;180)</td>
<td>171 (131;211)</td>
<td>155 (130;179)</td>
<td>126 (114;138)ab</td>
</tr>
<tr>
<td></td>
<td>Delta</td>
<td>6 (-18;29)</td>
<td>6 (-35;47)</td>
<td>-35 (-64;-7)ab</td>
<td>-51 (-65;-37)ab</td>
</tr>
</tbody>
</table>

Data given as mean with 95% confidence interval. VEH vehicle; NRT, non-responders to ACEi; IRT, intermediate responders to ACEi; RT, responders to ACEi. AUC, area under the curve. *: P < 0.05 versus ACEi NRT, #: P < 0.05 versus VEH.
ACE inhibitor resistance in chronic renal failure

Table 2. Renal and cardiac characteristics at autopsy

<table>
<thead>
<tr>
<th></th>
<th>VEH</th>
<th>ACEi NRT</th>
<th>ACEi IRT</th>
<th>ACEi RT</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>7</td>
<td>9</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>FGS (AU, max 200)</td>
<td>22 (13;30)</td>
<td>53 (12;94)</td>
<td>30 (3;58)</td>
<td>10 (5;16)</td>
</tr>
<tr>
<td>Interstitial α-smooth muscle actin (%)</td>
<td>6.0 (1.1;10.9)</td>
<td>5.7 (0.0;12.0)</td>
<td>4.5 (1.4;7.5)</td>
<td>1.6 (0.2;3.0)</td>
</tr>
<tr>
<td>Kidney/body weight ratio (mg/g)</td>
<td>5.7 (4.8;6.5)</td>
<td>5.9 (5.1;6.6)</td>
<td>5.4 (4.7;6.2)</td>
<td>5.7 (4.5;6.8)</td>
</tr>
<tr>
<td>Renal blood flow (ml/min/kg)</td>
<td>3.1 (2.1;4.1)</td>
<td>3.6 (2.0;5.2)</td>
<td>2.9 (2.3;3.6)</td>
<td>4.1 (2.2;6.0)</td>
</tr>
<tr>
<td>Heart/body weight ratio (mg/g)</td>
<td>3.3 (2.9;3.7)</td>
<td>3.4 (2.7;4.0)</td>
<td>3.1 (2.6;3.7)</td>
<td>3.1 (2.8;3.4)</td>
</tr>
<tr>
<td>Cardiac contractility +dP/dt_max (sec-1)</td>
<td>94 (82;105)</td>
<td>95 (86;104)</td>
<td>99 (91;106)</td>
<td>110 (105;116)</td>
</tr>
<tr>
<td>Cardiac relaxation -dP/dt_max (sec-1)</td>
<td>-85 (-95;76)</td>
<td>-81 (-91;-71)</td>
<td>-89 (-97;82)</td>
<td>-94 (-101;-88)</td>
</tr>
</tbody>
</table>

Data given as mean with 95% confidence interval. VEH, vehicle; NRT, non-responders to ACEi; IRT, intermediate responders to ACEi; RT, responders to ACEi. AUC, area under the curve; FGS, focal glomerulosclerosis. a: P< 0.05 versus ACEi NRT, b: P< 0.05 versus all other groups.

RESULTS

Group characteristics

All but one animal survived the surgical procedure of 5/6 nephrectomy (5/6NX) and gained weight during the experiment. Proteinuria increased from 18 (16-20) mg/24h at baseline to 154 (123-185) mg/24h six weeks after 5/6NX. After randomization, proteinuria further increased in vehicle treated animals from 134 (89-180) to 275 (190-361) mg/24h (P< 0.01) after 6 weeks. In contrast, proteinuria remained stable, ranging from 159 (120-197) at randomization to 156 (94-218) mg/24h in the groups treated for 6 weeks with ACEi.

Interestingly, a large variation in antiproteinuric response was observed in the ACEi group ranging from -70% to +343% (figure 1). In the responders, the AUC averaged -313 arbitrary units (AU), while in the intermediate responders and in the non-responders, the AUC averaged -148 AU and +128 AU, respectively. Notably, both the absolute proteinuria at randomization (table 1) and the AUC of proteinuria during week 1-6 were comparable between the groups (data not shown). Thus, defining response groups resulted in groups with marked differences in antiproteinuric efficacy of ACEi, yet with similar levels of proteinuria, creatinine clearance, and systolic blood pressure before the start of treatment (table 1). A weak relation was observed between creatinine clearance at week 6 and antiproteinuric response at week 12 (R^2= 0.20, P= 0.03, table 3).

Renal characteristics

Further differences in renal damage between response groups were assessed by measuring kidney weight, focal glomerulosclerosis, and α-SMA staining. While in the non-responders these markers were comparable to vehicle treated animals, a significant reduction was observed in the responders (table 2). Focal glomerulosclerosis was also significantly lower in the responders compared to the non-responders. Although a difference was observed in renal damage, creatinine clearance and renal blood flow were comparable between the groups at the end of the experiment. Thus, differential effects were observed with regard to structural renal damage, but not on renal hemodynamic parameters.
The antiproteinuric response to ACEi was not related to the level of proteinuria and blood pressure at the start of treatment (table 3).

<table>
<thead>
<tr>
<th>Table 3. Correlations</th>
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<tbody>
<tr>
<td>Antiproteinuric response (AUC) versus</td>
</tr>
<tr>
<td>Creatinine clearance wk 6</td>
</tr>
<tr>
<td>Creatinine clearance wk 12</td>
</tr>
<tr>
<td>Proteinuria wk 6</td>
</tr>
<tr>
<td>SBP wk 6</td>
</tr>
<tr>
<td>Delta SBP wk 6-12</td>
</tr>
<tr>
<td>Plasma lisinopril concentration wk 12</td>
</tr>
<tr>
<td>Renal lisinopril concentration wk 12</td>
</tr>
<tr>
<td>Average Urinary lisinopril excretion</td>
</tr>
<tr>
<td>Plasma ACE activity wk 6</td>
</tr>
<tr>
<td>Plasma ACE activity wk 12</td>
</tr>
<tr>
<td>Renal ACE expression wk 12</td>
</tr>
<tr>
<td>Renal ACE activity wk 12</td>
</tr>
</tbody>
</table>

Correlation between individual antiproteinuric effect of ACEi in AUC with parameters at wk 6 pre-treatment and wk 12 at autopsy in all ACEi treated rats.

**Hemodynamic and cardiac characteristics**

ACEi treatment did not lower systolic blood pressure in the non-responders, yet a significant reduction was found in the responders (table 1). Also, the antiproteinuric response correlated with the antihypertensive response measured as the decrease in systolic blood pressure (table 3). A similar trend was found for cardiac parameters measured at the end of the experiment, although not always significant. Cardiac contractility measured as \(+\frac{dP}{dt_{max}}/\Delta \text{mmHg}\) was comparable in the non-responders and in the vehicle treated rats, while in the responders contractility was significant higher compared to the other groups. Cardiac relaxation measured as \(-\frac{dP}{dt_{max}}/\Delta \text{mmHg}\) in the non-responders was comparable to the vehicle treated rats, while in the responders an improvement was observed (P= 0.06) compared to vehicle treated rats. Cardiac contractility and cardiac relaxation not only correlated with the reduction in systolic blood pressure during treatment (R²= 0.35, P= 0.002 and R²= 0.40, P= 0.001), but also with the antiproteinuric effect (R²= 0.40, P= 0.001 and R²= 0.23, P= 0.013). Thus, the response variation in the antiproteinuric effect of ACEi is reflected in systolic blood pressure and cardiac parameters as well.

**Pharmacodynamic variation in the renin angiotensin aldosterone system**

To investigate the pharmacological differences between the animals responsive and not responsive to ACEi therapy, renal, plasma, and cardiac ACE activity, and renal ACE expression were measured before and after treatment. At baseline, at the induction of disease (week 0), renal and plasma ACE activity were comparable in all groups as was renal ACE protein expression (figure 2). The non-responders showed
an increase in renal ACE activity between baseline and autopsy (P = 0.09), while this was not observed for the responders and the intermediate responders (Figure 2A). A similar pattern was observed for renal ACE protein expression in the groups: at baseline, at the induction of disease (week 0), renal ACE expression was comparable in all groups (Figure 2B). The non-responders showed an increase in renal ACE expression between baseline and autopsy (P = 0.02), while this was not observed for the responders and the intermediate responders (Figure 2B). In contrast to renal ACE activity, plasma and cardiac ACE activity was comparable in all groups after treatment (Figures 2C, D).

**Figure 2.** Effect of ACEi therapy on the Renin Angiotensin Aldosterone System. Panel A: renal ACE activity, panel B: renal ACE expression, panel C: plasma ACE activity, panel D: cardiac ACE activity. RT: responsive to ACEi therapy, IRT: intermediate responsive to ACEi therapy, NRT: not responsive to ACEi therapy. *: P < 0.05 or P = value given compared to baseline (week 0).
As the above results suggest a relationship between therapeutic response and renal ACE, this was further explored by correlation analysis. Renal ACE activity correlated with renal ACE protein expression ($R^2 = 0.23, P = 0.03$). The antiproteinuric AUC to ACEi correlated well with renal ACE expression and renal ACE activity at week 12 ($R^2 = 0.28$ and $P = 0.01$ and $R^2 = 0.43$ and $P < 0.001$ respectively, figure 3), but not with baseline values of these parameters obtained at week 0. In accord with the absence of differences in plasma and cardiac ACE activity of the groups, no correlations were found with the antiproteinuric AUC. Thus, the antiproteinuric effect on ACEi is related with renal ACE activity and renal ACE expression at week 12, but not with plasma and cardiac ACE activity.

**Pharmacokinetic variation: lisinopril concentration**

Differences between therapeutic efficacy may also be attributed to variations in pharmacokinetics, which was assessed by measurement of the lisinopril concentration in plasma, renal tissue and urine. The intake of lisinopril was comparable in all groups. Average water intake during the treatment period amounted $44 \pm 8$ ml/24h for the non-responders, $44 \pm 7$ ml/24h for the intermediate responders, and $40 \pm 4$ ml/24h for the responders. Plasma concentration of lisinopril at autopsy did not significantly differ between the three groups, although a trend towards higher plasma levels was observed in the responders compared to the non-responders (figure 4A, $P = 0.2$). Also, the three groups showed similar renal lisinopril levels (figure 4B). To further analyze differences in kinetics, the levels of urinary excretion during the treatment period were compared (figure 4C) and made clear that the average urinary lisinopril excretion showed a trend towards lower levels in the responders compared to the non-responders ($P = 0.19$, figure 4C).

![Figure 3. Correlation between antiproteinuric response and renal ACE parameters. panel A: renal ACE activity and panel B: renal ACE protein expression. Negative AUC denotes a decline in proteinuria.](image-url)
These data suggest that plasma lisinopril clearance is reduced in the responders compared to the non-responders and the intermediate responders. To further explore this, correlation analysis was performed using the average urinary lisinopril excretion during the treatment period, which correlated positively with the antiproteinuric AUC (figure 4D, table 3). However, the antiproteinuric AUC did not correlate with plasma and renal lisinopril levels obtained at autopsy (table 3). Collectively, these data indicate that a reduced renal lisinopril clearance is found in the responders compared to the non-responders.

**Figure 4.** Kinetic characteristics of lisinopril. Panel A: plasma lisinopril levels, panel B: renal lisinopril levels, panel C: average 24h urinary lisinopril excretion, panel D: correlation between antiproteinuric response and average 24h urinary lisinopril excretion. RT: responsive to ACEi therapy, IRT: intermediate responsive to ACEi therapy, NRT: not responsive to ACEi therapy. *: P< 0.05 compared to NRT.
**Discussion**

This study shows large inter-individual variation in the antiproteinuric response to ACE inhibitor therapy in 5/6 nephrectomized rats, which may be attributed to a combination of pharmacodynamic and pharmacokinetic differences between responders and non-responders. As a pharmacodynamic cause, we found high renal ACE protein levels and activity in non-responders. As a pharmacokinetic cause, we observed an increased lisinopril excretion in non-responders. Likely, both factors contribute to a reduced antiproteinuric therapeutic efficacy of ACEi, because of lower drug levels in the face of an increased amount of target protein. Moreover, differences in antiproteinuric efficacy were related to similar differences in effects on blood pressure and cardiac contractility parameters. Thus, it seems that the combination of renal pharmacodynamic and pharmacokinetic variation accounts for the inter-individual differences in response in 5/6 nephrectomized animals.

Thus far, the cause of the large inter-individual variation in response to ACEi therapy has not been thoroughly studied in rodent models of renal disease. In humans, it is known that the antiproteinuric effect of ACEi is dependent on sodium intake, ACE gene polymorphism, but probably not on initial proteinuria, blood pressure or GFR. In adriamycin nephrotic rats, the anti-proteinuric efficacy of ACEi was predicted by inter-individual differences in the extent of pretreatment renal damage. Our study strengthens the hypothesis that the therapy response is not dependent on initial proteinuria and blood pressure. The relation with GFR is still controversial, because only a low predictable relation could be found between creatinine clearance at start of treatment and antiproteinuric effect. It is unlikely that sodium intake may have caused the difference in therapy response, since all rats received the same diet.

In our study we identified two factors that account for the observed therapeutic resistance, the most obvious being a difference in pharmacokinetics. ACEi was administered to the rats in drinking water which was supplied *ad libitum*. While water intake, i.e. drug intake, was similar in responders and non-responders, urinary drug excretion was enhanced in non-responders. As lisinopril is mainly eliminated by the kidneys in the rat, the difference in urinary drug excretion in responders and non-responders indicate differences in renal pharmacokinetics. This is substantiated by the observation that the lowest steady state lisinopril levels were measured in non-responders following administration of an identical dose of drug by gavage 24h prior to collection of plasma samples. These findings are best explained by an increased renal clearance of lisinopril in non-responders. This increased renal clearance is not caused by a difference in glomerular filtration since GFR, estimated by creatinine clearance, was similar in responders and non-responders. The ionisation of lisinopril is not pH dependent, therefore a change in urine pH would not influence excretion. Moreover, the plasma protein binding of lisinopril is 0%, therefore a variation in plasma protein levels caused by proteinuria cannot account for the observed response variation. Consequently, the pharmacokinetic difference between the groups is most likely explained by differences in tubular handling of the drug.

A second –pharmacodynamic- factor explaining the non-response to ACEi in 5/6NX is an increased renal ACE activity. As the increased ACE activity in the NRT groups was paralleled by an increase in renal protein levels of ACE, the difference is most likely explained by differences in the regulation of renal ACE expression. Tissue ACE expression is regulated by Angiotensin II levels via a negative feedback system. Under ACEi therapy, not much is known about this feedback system. Of note is that ACE expression remains at baseline levels in the responders, but increases in the non-responders. The nature of the regulation of ACE remains still obscure. It seems unlikely that it is related to different levels of...
renal ACEi, because feedback would result in an increase in ACE expression or activity in the group with the most effective inhibition of the enzyme, i.e. the RT group. Possibly, the differential regulation of ACE expression in responders and non-responders is related to the ACE polymorphism in the rat\textsuperscript{27,28}. At autopsy, a correlation was observed between the antiproteinuric effect of ACEi and both renal ACE activity and renal ACE expression: the larger the antiproteinuric effect, the lower both the renal ACE activity and the renal ACE expression. Unfortunately, in this experimental setting, it is not possible to measure renal ACE activity and ACE expression at randomization, the time point at which disease had already developed. Therefore, we would have been obliged to collect renal tissue at that time point by renal biopsy, which is not preferable in 5/6 nephrectomized rats, because of the already reduced mass renal tissue. However, we do have circumstantial arguments that renal ACE activity at randomization is not different between the groups. Renal ACE activity at week 12 relates to proteinuria at week 12 (R\textsuperscript{2}= 0.46, P= 0.09) in the vehicle group and no differences were observed in renal ACE activity between the groups at week 0. Therefore, as proteinuria was comparable in all groups at randomization, it is conceivable that renal ACE activity is not different between the groups as well.

In line with the above data, previous studies have suggested that renal ACE expression governs the development of renal damage. In adriamycin nephrotic rats it was shown that the naturally occurring variation in baseline renal ACE activity in outbred animals predicts renal damage after the induction of disease\textsuperscript{34}. We have evidence that this is true for 5/6NX in outbred animals as well\textsuperscript{15}. In the vehicle group, we found a correlation between baseline renal ACE activity and focal glomerulosclerosis (R\textsuperscript{2}= 0.64, P= 0.056). Therefore, baseline renal ACE activity might be predictive for renal damage in 5/6NX rats. Consequently, the same mechanism of the increased renal ACE activity in non-responders might provoke accelerated renal damage explaining the variation in therapy response. It is not known whether increased dosage of ACEi is effective to break through this response resistance in non-responders. This should be examined, particularly because therapy response in the adriamycin nephrotic rat model did not improve by dose increase or combining ACEi with an angiotensin II receptor blocking agent\textsuperscript{6}, whereas in recent clinical studies it is clearly shown that increasing doses of ACEi or Angiotensin II receptor blocker (to even extremely high dose) enhances the antiproteinuric response without affecting the blood pressure\textsuperscript{35-37}.

Renal ablation by 5/6NX is a hypertensive model provoking both renal and cardiac damage\textsuperscript{35-37}. The cardiac damage is responsive to ACEi as well\textsuperscript{98}. Therefore, hemodynamic and cardiac characteristics were measured to investigate differences in therapy response. Interestingly, the difference in the response was not only observed for the kidney but also for systemic (blood pressure) and cardiac parameters, and more interestingly, renal non-responders also showed cardiac and systemic non-response. Since we found similar ACE activity in the heart tissue and in plasma of responders and non-responders, it appears that the renal response variation determines the cardiac and systemic response variation. Recent studies suggest a mutual interaction of organ damage in the heart and the kidney when renal damage occurs\textsuperscript{59} and that this vicious circle is responsive to ACEi therapy\textsuperscript{18}, although it is unclear whether ACEi affects primarily the heart, the kidney or both. The present data may indeed indicate that by improving the kidney with ACEi, this has also a beneficial effect on the heart.
CONCLUSION
In conclusion, our results show that therapeutic resistance to ACEi is related to a difference in the combination of renal pharmacodynamic and pharmacokinetic characteristics in non-responders, primarily consisting of increased renal ACE expression and higher ACEi clearance. Future experiments are needed to define adequate therapeutic measures to overcome therapeutic resistance to ACEi. In particular, the effects of increasing the dosage in non-responders should be evaluated.

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