Renal endothelial function is associated with the anti-proteinuric effect of ACE inhibition in 5/6 nephrectomized rats

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Experimental animals. Male Wistar rats [Hsd.Cpb.Wu (n = 29); 200–250 g; Harlan; Horst, The Netherlands] were used and housed under standard conditions (day/night rhythm 12:12 h, group housing in macrolon cages) approved by the institutional animal ethical committee. The rats had free access to food and drinking water and received a normal diet (RMH-B, 2181; ABDiets, Woerden, the Netherlands). After arrival in our laboratory, the rats had an acclimatization period for 1 wk to get used to their new environment. After this period, the rats were trained for SBP measurements (i.e., accustomed to immobility in a warmed restrainer for at least 10 min), because stress would cause elevation of blood pressure.

All animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Committee for Animal Experiments of the University of Groningen (DEC4162B).

Surgery: 5/6Nx. By laparotomy and under anesthesia with 3% isoflurane in N2O/O2 (2:1), rats (n = 22) underwent 5/6Nx, as described previously (11). Briefly, by removing the right kidney and by ligating two or three branches of the renal artery of the left kidney,
leading to infarction of approximately 2/3 of this kidney. Sham animals (n = 7) underwent the same procedure with manipulation and partial decapsulation of the kidneys, but without Nx or arterial ligation. Postoperatively, rats received a subcutaneous injection of diluted buprenorphine (Temgesic; 0.01 mg/kg) for analgesic purposes and were allowed to recover for 1 wk.

Study design. All rats survived the surgical procedures and were followed for up to 15 wk thereafter (Fig. 1). SBP and 24-h urinary protein excretion were determined weekly. At baseline (time t = 0, i.e., shortly before surgery), after 6 wk (t = 6, randomization), and at 15 wk (t = 15, termination), blood samples were collected for the determination of plasma creatinine. Six weeks after surgery, 5/6Nx rats were stratified based on proteinuria and randomized in untreated (no treatment-5/6Nx, n = 11) and treated (lisinopril-5/6Nx; n = 11) rats. Sham rats (n = 7) were left untreated. Lisinopril was dissolved in the drinking water at a concentration of 2.5 mg·kg body wt−1·day−1.

**In vitro perfusion setup.** Renal interlobar arteries obtained from the exirrigated right kidney were cleaned from perivascular tissue and transferred to an arteriograph system for pressurized arteries (Living Instrumentation, Burlington, VT), as described previously (6). Arterial segments were cannulated on glass micropipettes, and the vessel chamber was continuously recirculated with warmed (37°C) and oxygenated (5% CO₂ in O₂) Krebs solution with a pH of 7.4. An inverted light microscope attached to a video camera and video dimension analyzer was used to continuously register lumen diameter and thickness of arterial wall.

**ACH-mediated relaxation of renal interlobar arteries.** Intraluminal pressure was set at 60 mmHg. Arteries were allowed to equilibrate for 45 min and checked for smooth muscle and endothelium viability by stimulation with single concentrations of phenylephrine (PE, 3 × 10⁻⁷ mol/l) and ACh (3 × 10⁻⁸ mol/l). After washing and renewed stabilization, arteries were preconstricted with PE (10⁻⁶ mol/l) before endothelium-dependent relaxation was assessed by administering cumulative doses of ACh (10⁻⁹ to 10⁻⁶ mol/l) to the recirculating bath.

**Follow-up measurements of proteinuria, blood pressure, and serum creatinine.** Urinary protein excretion was measured in samples of 24-h urine collected when the rats were put in metabolic cages for 24 h on a weekly basis for 15 wk. Urinary protein concentration was determined by nephelometry (Dade Behring III). Weekly SBP measurements were carried out as the mean of three consecutive measurements obtained in conscious animals with tail-cuff plethysmography (IITC Life Science, Woodland Hills, CA). Creatinine concentration was measured colorimetrically (Chema Diagnostica, Jesi, AN, Italy).

**Solutions and drugs.** Vessel segments were perfused with Krebs solution of the following composition (in mmol/l): NaCl 120.4, KCl 5.9, CaCl₂ 2.5, MgCl₂ 1.2, NaH₂PO₄ 1.2, glucose 11.5, NaHCO₃ 25.0. Lisinopril was purchased from Astra-Zeneca (The Netherlands). All other compounds were purchased from Sigma (St. Louis, MO).

**Data analysis.** Data were expressed as means ± SE; n values represent the number of investigated rats, as well as the number of investigated arteries, since one artery segment per rat was used for the same protocol. Concentration-response (CR) curves to ACh were expressed as percentage of preconstriction to PE. Area under the endothelial function curve (AUC) was determined (SigmaPlot 13) and expressed in arbitrary units. Ordinary least squares were used for regression analysis (SigmaPlot 13). For comparison of proteinuria and blood pressure over time, repeated-measures ANOVA was used with the Greenhous-Geisser correction (SPSS), as recommended by Ludbrook (9). Unless stated otherwise, additional differences for vascular parameters, proteinuria, SBP, body and organ weights, water intake, and urinary output were determined by ANOVA following least

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Table 1. Rat characteristics at baseline (t = 0) and at 6 wk following surgery (t = 6)

<table>
<thead>
<tr>
<th></th>
<th>Sham (n = 7)</th>
<th>5/6Nx (n = 22)</th>
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<tr>
<td></td>
<td>t = 0: baseline</td>
<td>t = 6: stratification</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>321 ± 4</td>
<td>427 ± 9</td>
</tr>
<tr>
<td>Nephrectomized kidney weight, g</td>
<td>1.56 ± 0.05</td>
<td>123 ± 2</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>124 ± 6</td>
<td>137 ± 5</td>
</tr>
<tr>
<td>Water intake, ml/24 h</td>
<td>21 ± 2</td>
<td>26 ± 2</td>
</tr>
<tr>
<td>Urine production, ml/24 h</td>
<td>10 ± 1</td>
<td>14 ± 2</td>
</tr>
<tr>
<td>Proteinuria, mg/24 h</td>
<td>20 ± 3</td>
<td>26 ± 2</td>
</tr>
<tr>
<td>Plasma creatinine, μmol/l</td>
<td>46 ± 5</td>
<td>47 ± 4</td>
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Values are means ± SE; n, no. of rats. Nx, nephrectomy. *P < 0.05, **P < 0.01, and ***P < 0.001 for 5/6Nx vs. sham at the moment of stratification (t = 6 wk).
significant difference post hoc test (SPSS). Differences were considered significant at *P* < 0.05 (two-tailed).

### RESULTS

**Rat characteristics and renal artery function at baseline.** Body weight, water intake, urinary output, plasma creatinine, urinary protein excretion, and SBP did not significantly differ between the rats allocated to sham surgery and those allocated to 5/6Nx at the start of the study (*t* = 0) (Table 1). Interlobular renal arteries were isolated from extirpated kidneys during 5/6Nx and studied for ACh-induced endothelium-dependent relaxation. The data in Table 2 show that the features of the vessels and ACh CR curves did not significantly differ at baseline between the animals later stratified to ACEi treated and untreated groups. Figure 2A shows the average CR curve to ACh for all 5/6Nx rats and depicts how the AUC was determined. Figure 2B, in which the individual ACh-AUC is plotted for all rats, shows that baseline ACh-induced relaxation varied considerably among normal rats and was comparable between the animals later stratified to the untreated and treated groups. The data confirm that the group of rats stratified for later treatment with ACEi was a representative portion of normal healthy rats.

**Development of high blood pressure and proteinuria following 5/6Nx.** Both SBP and urinary protein excretion progressively increased in the first weeks following 5/6Nx (Fig. 3). Additionally, water intake, urine production, and plasma creatinine levels at *t* = 6 wk were significantly higher in 5/6Nx rats compared with sham (Table 1). Unlike proteinuria, SBP plateaued at week 5–6 to remain steadily elevated thereafter in the untreated 5/6Nx rats (Fig. 3B). Figure 4 shows a scatterplot of SBP vs. proteinuria at *t* = 6 wk for all rats; analysis suggested a best fit by a second-order polynomial regression. When the relationship was analyzed for rats with SBP < 160 mmHg (which covered the range and maximum value observed in sham rats) and SBP ≥ 160 mmHg, SBP and proteinuria positively correlated in the rats with the higher blood pressure (see inset in Fig. 4).

ACEi treatment with lisinopril reduces blood pressure and slows proteinuria. Before treatment, no significant differences were found in any of the measured parameters between 5/6Nx rats stratified to receive ACEi treatment with lisinopril or to remain untreated (Table 3). This confirms that the group of rats allocated to ACEi treatment was a representative portion of 5/6Nx rats. After week 6, renal disease further progressed in untreated 5/6Nx rats as evidenced by the increase in proteinuria levels, while SBP remained steadily elevated and did not further increase. ACEi treatment substantially reduced proteinuria compared with untreated 5/6Nx rats, although the levels were still higher than in sham animals. Furthermore, the development of proteinuria was just delayed after lisinopril, with the maximum decrease at *t* = 6–8 wk (~35%), but not prevented as it increased again thereafter (Fig. 3A). Lisinopril also reduced SBP, with the maximum decrease at *t* = 6–10 wk (~25%), after which it remained stable at normotensive level (Fig. 3B). The data show that, while ACEi treatment normalized blood pressure, it only slowed down but did not prevent the development of proteinuria. As for the increased plasma creatinine in 5/6Nx rats at *t* = 6 wk, the levels did not further increase after lisinopril (72 ± 15 μmol/l at *t* = 15 wk) and were no longer significantly different from those in sham rats (57 ± 5 μmol/l, *P* = nonsignificant) at *t* = 15 wk (Table 3).
Baseline renal artery function predicts the ACEi effect on proteinuria and blood pressure. To analyze the relationship of renal artery function at baseline with the anti-hypertensive and anti-proteinuric effects of ACEi treatment thereafter, we considered the relative changes from \( t = 6 - 8 \) wk (i.e., when maximum decrease in proteinuria was observed), from \( t = 6 - 10 \) (i.e., when maximum decrease in SBP was observed) and from \( t = 6 - 15 \) (i.e., the overall effect during the entire observation period) in 5/6Nx rats treated with lisinopril. Hence, changes were expressed as the percentage difference from starting values at \( t = 6 \) (see Fig. 5) and calculated as the AUC (in arbitrary units) for the selected time frames and used for univariate linear regression analysis with baseline renal artery ACh dilation (as it is reported in Fig. 2B) in the group of 5/6Nx rats treated with lisinopril. The analysis showed that the decrease in proteinuria was highly significantly correlated with baseline ACh dilation, such that individuals with smaller renal artery endothelium-dependent dilation at baseline showed a larger anti-proteinuric response to lisinopril (Fig. 6A). This seemed also true for the anti-hypertensive effect of lisinopril, but only when the longer treatment period was considered, i.e., the correlation between baseline ACh dilation and blood pressure-lowering effect of ACEi treatment was significant for the time frame \( t = 6 - 15 \) wk, but not for \( t = 6 - 8 \) and \( t = 6 - 10 \) (Fig. 6B).

**DISCUSSION**

Here we found that normal physiological variation in renal artery endothelial vasodilatory function in otherwise healthy individuals had a predictive value for the future response to ACEi treatment with lisinopril in a setting of renal damage development (i.e., proteinuria) and hypertension following subtotal Nx in rats. The nature of the relationship between both, i.e., ACh-induced renal artery dilation at baseline negatively correlated with the decrease in proteinuria and SBP in lisinopril treated animals, suggests that individuals with decreased endothelial vasodilatory function respond better to ACEi therapy.
Development of hypertension and proteinuria following 5/6Nx. The so called 5/6Nx model, where one kidney is removed and 2/3 of the remaining kidney is ablated, mimics the progressive renal failure after loss of renal mass in humans. The 2/3 ablation may be achieved either by polar removal of renal tissue or ligation of branches of the left renal artery to cause interruption of the blood supply and infarction of the renal tissue (1, 2, 5). Approaches with infarction, i.e., as in the present study, typically are associated with more severe hypertension and proteinuria than those with only excision, likely due to distinct upregulation of the renin angiotensin system in the peri-infarct zone after ligation (18). Consistent herewith, SBP in the present study increased by ~35% on average at 6 wk following 5/6Nx, after which it remained steadily elevated. Furthermore, the increase in blood pressure was accompanied by an increase in urinary protein excretion, albeit with a slight delay. This, together with the observed positive correlation between SBP, particularly at the higher levels >160 mmHg, and proteinuria at 6 wk after 5/6Nx (Fig. 4) may confirm a role of hypertension in renal damage development (following renal mass reduction, among others).

A similar relationship was also found by Griffin et al. (4) who studied susceptibility to nephropathy after nitric oxide (NO) inhibition in Sprague Dawley (SD) rats from two major suppliers. In that study, Nω-nitro-L-arginine methyl ester treatment was started 2 wk after 3/4 normotensive Nx and lasted for a period of 4 wk. In SD rats obtained from Charles River, Nω-nitro-L-arginine methyl ester failed to induce proteinuria and glomerular injury, despite moderate hypertension (~144 mmHg). By contrast, SD rats obtained from Harlan developed greater SBP (~169 mmHg) and glomerular injury, and the both were positively correlated. The authors noticed that substantial differences exist in the response to NO synthesis inhibition between and within rodent strains and concluded that those strains (hence, individuals) sharing the phenotype of a blunted blood pressure response to NO synthesis inhibition also appear to exhibit a relative resistance to renal damage and its progression (4). Consistent with such findings, our laboratory (6) previously found that intrarenal artery endothelial (NO-mediated) vasodilatory function of the surgically removed kidney during ablation in normal Wistar rats predicted the individual’s susceptibility to injury in the remnant kidney of the same rat in a normotensive setting. In the present study, however, baseline intrarenal artery endothelial vasodilatory function did not significantly correlate with the level of proteinuria at 6 wk following 5/6Nx (data not shown). The major difference between both studies is that blood pressure was increased in the present but not the former study, despite employment of similar methods and rat strain. A phenotypical change in blood pressure response to 5/6Nx following changes in external and/or local housing conditions over time might explain the disparity between our earlier and present study,

Table 3. Characteristics of 5/6Nx rats at the onset of treatment (t = 6; stratification) and at the end of the study period (t = 15; termination)

<table>
<thead>
<tr>
<th></th>
<th>5/6Nx Untreated (n=11)</th>
<th>5/6Nx Lisinopril (n=11)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>t = 6: stratification</td>
<td>t = 15: termination</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>422 ± 13</td>
<td>493 ± 16</td>
</tr>
<tr>
<td>Remnant kidney weight, g</td>
<td>—</td>
<td>2.27 ± 0.12</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>164 ± 10</td>
<td>171 ± 6</td>
</tr>
<tr>
<td>Water intake, ml/24 h</td>
<td>44 ± 3</td>
<td>47 ± 4</td>
</tr>
<tr>
<td>Urine production, ml/24 h</td>
<td>23 ± 2</td>
<td>33 ± 4</td>
</tr>
<tr>
<td>Proteinuria, mg/24 h</td>
<td>82 ± 19</td>
<td>317 ± 44</td>
</tr>
<tr>
<td>Plasma creatinine, μmol/l</td>
<td>78 ± 5</td>
<td>152 ± 71</td>
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</table>

Values are means ± SE; n, no. of rats. Nx, nephrectomy. *P < 0.05, **P < 0.01, and P < 0.001 for 5/6Nx lisinopril vs. 5/6Nx untreated at termination (t = 15 wk).
Initiation of ACEi treatment with lisinopril reduced proteinuria and SBP, reaching maximum response to ACEi treatment. In such case, proteinuria would have just been predicted by baseline renal artery endothelial vasodilatory function, such that individuals with decreased endothelial vascular function, such as indicated.

Although this has not been the subject of study. On the one hand, the lack of a clear explanation to account for the phenotype of the blunted blood pressure response observed previously complicates and limits a comparison to the present results. When taken for granted, however, the combined results leave open that the predictive value of renal endothelial vasodilatory function for susceptibility after subtotal Nx may be modified or overruled by concomitant development of hypertension.

The fact that blood pressure reduction persisted while the anti-proteinuric effect was temporary may suggest that ACEi exert an independent control (to some extent) over proteinuria and blood pressure. Hence, it could be argued that the initial high blood pressure upon 5/6Nx in the present study may have triggered some (e.g., glomerular) lesions that eventually lead to proteinuria, even though blood pressure was normalized during ACEi treatment. In such case, proteinuria would have just been a delayed consequence of hypertension. Conversely, 5/6Nx in our laboratory’s previous study also resulted in proteinuria, yet in the absence of high blood pressure (6). The latter seems to leave room for nonhemodynamic effects contributing to angiotensin 2 [or angiotensin 2 type 1 (AT1) receptor activity?]-induced proteinuria as well (3), eventually because of the (individual differences in) renin-angiotensin system (RAS) activity with endothelial vascular function interaction, as we tend to believe. Nevertheless, ACEi treatment was not part of our laboratory’s previous study, and it should be acknowledged that evidence for the alleged RAS-activity link with endothelial function, although circumstantial, is not sufficiently refined to substantiate such claim here.

Baseline renal artery function predicts the anti-proteinuric response to ACEi treatment. Initiation of ACEi treatment with lisinopril reduced proteinuria and SBP, reaching maximum decreases at 2 and 4 wk after the onset of treatment, respectively. Unlike blood pressure, which then remained stable at normotensive level throughout follow-up, the anti-proteinuric effect was temporary in that proteinuria increased again thereafter. The latter is in agreement with a previous study in 5/6Nx rats in which responders, intermediate responders, and nonresponders to ACEi therapy were identified based on their anti-proteinuric response, with the average anti-proteinuric response for the group as a whole being comparable to that in the present study (17). The fact that blood pressure reduction persisted while the anti-proteinuric effect was temporary suggests that ACEi may exert a double, probably independent control over proteinuria and blood pressure.

Importantly, the anti-proteinuric effect of lisinopril was predicted by baseline renal artery endothelial vasodilatory function, such that individuals with decreased endothelial vasodilatory function responded better to ACEi therapy. It should be noted that this concerned only the variation in proteinuria during ACEi treatment, but not the absolute values (data not shown). At the same time, we have to acknowledge that a failure to detect such an association in our present study does not mean that it does not exist. Within these limitations, our findings may strengthen a concept in which endothelial vasodilatory function responded better to ACEi therapy.
We also know that the RAS, via activation of reactive oxygen species generating NADPH oxidases, is closely related to endothelial (dys)function (7, 12). In particular, reactive oxygen species (superoxide radicals), which neutralize NO, is thought to underlie reduced NO availability and lead to impaired endothelium-dependent relaxation (11). Collectively, the above suggests that individual variation in local renal RAS activity, as may be observed in normal healthy subjects, may very well influence individual endothelial function in the renal preglomerular vasculature and actually account to some extent for the physiological variation in endothelial function in normal healthy subjects. Therefore, it may be conceived that individuals with reduced endothelial relaxation function based on a higher individual RAS activity might actually benefit more from ACEi therapy.

To summarize, we found that, in 5/6Nx animals, renal artery endothelial vasodilatory function, as well as the development of renal damage and the response to ACEi, displays large interindividual variation. Since a similar variability in the anti-proteinuric and renal-protective response to ACEi is also present in the clinical setting (11, 14), it seems of relevance to identify early responders and nonresponders and get more insight in the underlying mechanisms to improve therapeutic results. The present findings suggest that physiological variability in renal endothelial vasodilatory function provides prognostic information on ACEi therapeutic responsiveness. In fact, in the present study, baseline renal artery endothelial vasodilatory function predicted the anti-proteinuric response to ACEi, as well as the anti-hypertensive response at the longer term. In particular, our results suggest that individuals with decreased endothelial vasodilatory function may respond better and profit more from ACEi therapy. Nevertheless, these findings need further research for confirmation and causal understanding. For example, future investigations for the effect of other blood pressure-lowering interventions may provide further insight regarding the exclusivity (or not) of the present findings with respect to RAS inhibition. Such studies may also include investigation of the role of the AT1 receptor in modulation of interindividual variation of myogenic responses of intrarenal arteries to further investigate whether individual differences in the local RAS (at baseline) may contribute to the finding that there are good and poor responders to ACEi. The present results may provide fuel for such future studies as a means to eventually better understand the mechanisms of variable individual response of CKD patients to ACEi therapy with means to optimize such treatments.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: S.V. and D.D.Z. conception and design of research; S.V. and P.O. performed experiments; P.V. analyzed data; P.V. drafted manuscript; P.O. interpreted results of experiments; L.E.D. prepared figures; R.H.H. and H.B. edited and revised manuscript; R.H.H. and H.B. approved final version of manuscript.

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