Chapter 5

Selective impairment of myogenic constriction and endothelial function of small renal arteries precedes the development of renal damage in the hypertensive Fawn-Hooded rat

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Abstract

Background: Chronic kidney disease is associated with abnormal regulation of arterial tone throughout various vascular beds. It is however unknown whether generalized vascular dysfunction precedes the development of kidney disease. We studied myogenic constriction and endothelium-mediated dilatory responses in two inbred Fawn-Hooded (FH) rat strains, one of which (FHH) spontaneously develops hypertension, proteinuria and glomerulosclerosis, whereas other (FHL) does not.

Methods: Small renal, mesenteric resistance arteries and aorta isolated from FH rats prior to (7 weeks old) and following the development of mild proteinuria (12 weeks old), were mounted in perfused and isometric set-ups, respectively. Myogenic response, acetylcholine-induced endothelium-dependent relaxation and the contribution of nitric oxide (NO), cyclooxygenase (COX)-derived prostaglandins and endothelium-derived hyperpolarizing factor (EDHF) were studied using the inhibitors L-NMMA, indomethacin and charybdotoxin+apamin, respectively.

Results: In small renal arteries, markedly impaired myogenic reactivity and endothelial dysfunction due to excessive COX1-mediated production of constrictive prostaglandins were selectively present in FHH as compared to FHL even prior to the development of proteinuria. In contrast, myogenic reactivity was intact in mesenteric resistance artery of FHH. In addition, mesenteric endothelial dysfunction was observed attributed to the loss of EDHF. Renal myogenic and peripheral EDHF alterations were further attenuated after the development of proteinuria. Aortic reactivity did not differ between FHL and FHH at the time points studied.

Conclusion: The present study shows that vascular dysfunction in both small renal and systemic arteries precedes renal end-organ damage in a spontaneous model of hypertension-associated renal damage. Renal and peripheral vasomotor mechanisms are affected heterogeneously and differentially modulated by renal disease. These early vascular changes might be potentially involved in the increased susceptibility of FHH rats to renal injury.
Introduction

In chronic kidney disease, a progressive deterioration in renal function is associated with abnormal regulation of arterial tone, both on the level of endothelium and vascular smooth muscle. A growing body of evidence indicates that even patients with microalbuminuria, an earliest marker of renal structural damage, exhibit generalized endothelial dysfunction in both renal and systemic vascular beds. In fact, it has been suggested that endothelial dysfunction might precede the development of microalbuminuria, thus potentially representing a determinant of renal disease progression. Endothelium-dependent relaxant responses, a surrogate of endothelial function, are mediated by the release of vasoactive factors, such as nitric oxide (NO), cyclooxygenase-derived prostaglandins (PGs) and the yet unidentified endothelium-derived hyperpolarizing factor (EDHF). We have previously shown, that the interindividual variability in endothelium-dependent reactivity of intrarenal arteries, including NO-, PGs- and EDHF-mediated responses, among healthy rats of an outbred Wistar rat strain predicts their susceptibility to subsequent renal damage induced by renal mass reduction or nephrotoxic drug. This suggests that endothelial function might be one of the factors governing the susceptibility to experimental renal end-organ damage. In addition to experimental models, specific inbred animal strains have been described spontaneously developing progressive renal disease. Fawn-hooded (FH) rat provides a genetically well-defined model comprised of two inbred strains with different occurrence of renal injury. Hypertensive fawn-hooded rats (FHH) spontaneously develop moderate hypertension, proteinuria and severe glomerulosclerosis at a young age, subsequently followed by progressive renal failure. In contrast, Fawn-hooded rats with low blood pressure (FHL) seem to be resistant to the development of hypertension and renal damage. It has been proposed that altered vascular-smooth muscle-mediated reactivity to intraluminal pressure, termed myogenic response, in preglomerular arteries might be responsible for different sensitivity of these strains to renal injury. Yet, the data comparing renal myogenic response in FHL and FHH rats provide inconsistent results, whereas the role of endothelial reactivity is unknown. In the present study we aim to define the role of vasomotor changes in the course of spontaneous hypertension-associated renal disease. First, we explored whether renal vascular dysfunction precedes the development of renal damage. To this end, in animals prone (FHH) and resistant (FHL) to renal damage, we compared endothelium-mediated (NO-, PGs and EDHF-dependent) and myogenic responses of small renal arteries at early age, prior to the appearance of renal damage. In addition, we explored whether renal vascular changes reflect generalized vasomotor dysfunction by studying endothelial and myogenic responses in small resistance (mesenteric) and large conduit arteries (aorta). Finally, we evaluated the observed vascular changes in time, by investigating vasomotor reactivity in animals at the older age, when mild proteinuria had already developed.
Materials and methods

Animals and in vivo measurements
Experiments were performed in young male FHL and FHH rats at week 7 and week 12 after birth (n= 9-12 per strain and per time point). In FHH rats, this time frame represents the ages in which none and minor proteinuria is detected, respectively. All animals were bred at the animal facilities of Erasmus University, Rotterdam, the Netherlands and housed under standard conditions in animal facility of University of Groningen, the Netherlands receiving food and water ad libitum. Short before reaching their target age, animals were put in metabolic cages to measure fluid intake and urine output. Urinary protein excretion was determined by nephelometry (Dade Behring III, Mannheim, Germany) in 24-hour urine samples. Subsequently, animals were anesthetized by 2% isoflurane in N₂O/O₂ (2:1), the right carotid artery was cannulated, and systolic and diastolic blood pressure was measured by a pressure transducer catheter (Millar Instruments, Germany) in the aortic root. Following these measurements, blood was drawn via abdominal aorta. Subsequently, mesenterium, kidneys and thoracic aorta were harvested for the analysis of vascular function and end-organ damage. All animal experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Committee for Animal Experiments of the University of Groningen.

Determination of renal damage
Plasma and urine creatinine was measured by means of a photometric assay with the Jaffé method without deproteinization (DiaSys Diagnostic Systems, Holzheim, Germany) and creatinine clearance was calculated as creatinine clearance = (urine creatinine x urine flow) / (plasma creatinine x bodyweight). Paraffin embedded kidneys were cut in 3 µm sections and stained with periodic acid Schiff (PAS) and the incidence of focal glomerulosclerosis (FGS) score was microscopically evaluated according to standard procedures as described previously.¹⁸

Vascular reactivity of small renal and systemic resistance arteries
Small renal (interlobar) arteries and third-order branches of superior mesenteric arteries were cleaned from perivascular tissue and transferred to an arteriograph system for pressurized arteries (Living System Instrumentation, Burlington, VT, USA) as described previously.⁹ Artery 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.

Myogenic reactivity of small renal and systemic resistance arteries
Intraluminal pressure was set at 80 mmHg, arteries were allowed to equilibrate for 40 minutes and checked by a single dose of phenylephrine (PE, 3x10⁻⁷ mol/l) and
acetylcholine (ACh; 3x10^{-5} mol/l), for smooth muscle and endothelium viability, respectively. Following a wash out, intraluminal pressure was decreased to 20 mmHg and myogenic reactivity was studied by obtaining active pressure-diameter curves over a pressure range of 20-160 mmHg in steps of 20 mmHg. Each pressure step was maintained for 5 minutes to reach the stable contractile response. Following the myogenic protocol, preparations were washed with Krebs solution and employed for investigation of endothelial function as described below. Thereafter, calcium containing Krebs solution was exchanged for calcium-free Krebs solution supplemented with ethyleneglycol-bis-(b-aminoethylether)tetraacetic acid (EGTA, 2 mmol/l) and passive pressure-diameter curves were obtained over the same 20-160 mmHg pressure range.

To explore the role of endothelium in myogenic tone regulation, the effect of endothelium removal was investigated in additional renal arteries (n=10 for each strain) isolated from 12 weeks old animals. Endothelium was removed by perfusing the preparation with 5 ml of air and endothelial removal was confirmed by the absence of dilative response to ACh (3x10^{-5} mol/l) following a submaximal pre-constriction with PE (3x10^{-7} mol/l). Subsequently to the wash-out, active and passive myogenic curves were recorded as described above.

**Endothelium-dependent relaxation of small renal and systemic resistance arteries**

Following the measurement of active myogenic curves, intraluminal pressure was set to 80 mmHg and arteries were washed and stabilized for 20 minutes. Because the level of spontaneous tone was not sufficient for the subsequent relaxation studies, arteries were pre-constricted with phenylephrine (3x10^{-7} - 10^{-6} mol/l) to 50-60% of initial baseline diameter. Endothelium-dependent relaxation was assessed by administering cumulative doses of acetylcholine (ACh; 10^{-9} - 3x10^{-5} mol/l) to the recirculating bath. After the construction of a full ACh concentration-response curve and wash out, the response to ACh was studied in the same artery in the presence of indomethacin (10^{-5} mol/l) to inhibit prostaglandins (PGs) production. Subsequently, the same procedure was repeated in the combined presence of indomethacin and the nitric oxide (NO) production inhibitor Nω-monomethyl-L-arginine (L-NMMA, 10^{-4} mol/l). In some arteries of both vascular types (FHL and FHH, n=4 each), we found that this remaining relaxation in the presence of indomethacin and L-NMMA was caused by the release of endothelium-derived hyperpolarizing factor (EDHF), as it was completely attenuated by the combination of the potassium channels blockers charybdotoxin (chtx, 10^{-7} mol/l) and apamin (apa, 5x10^{-7} mol/l). By analyzing the above-mentioned protocols of endothelium-dependent relaxation, the contribution of all three mediators (PGs, NO and EDHF) to endothelial relaxation was calculated as a difference between Area Under the Curve (AUC) of respective ACh-concentration-response curves.

**Involvement of cycloxygenase (COX) pathway in endothelium-dependent contractions of small renal arteries**

To investigate the underlying mechanisms of prostanoid-dependent endothelium-mediated contractions observed in small renal arteries of FHH rats, in separate set of arteries (n=6),
ACh-concentration responses were obtained in presence of either the COX-1 selective inhibitor valeryl salicylate (VAS, $10^{-4}$ mol/l), the COX-2 selective inhibitor NS398 ($10^{-6}$ mol/l), the TXA$_2$/PGH$_2$ receptor antagonist SQ29548 ($10^{-6}$ mol/l) or the superoxide scavenger superoxide dismutase (SOD, 50 U/ml).

**General smooth muscle reactivity of small renal and systemic arteries**

Additional arteries were used to control for the potential variation in depolarization-and receptor-mediated smooth muscle reactivity. After a stabilization period, concentration-response contractile curves were obtained using KCl (20-120 mmol/l) and phenylephrine ($10^{-8}$-$10^{-5}$ mol/l) with a wash out period between the protocols. Additionally, concentration-response curves to the direct smooth muscle vasodilator sodium nitroprusside (SNP, $10^{-9}$-$3x10^{-5}$ mol/l) were constructed after submaximal preconstriction with phenylephrine ($3x10^{-7}$-$10^{-6}$ mol/l).

**Vascular reactivity of isolated aortic rings**

The thoracic aorta was cleaned from the connective tissue and cut into 2 mm rings, which were mounted in isotonic contraction organ baths filled with aerated, warmed Krebs solution and subjected to 14 mN preload. After one hour stabilization period, arteries were stimulated by KCl (60 mmol/l) to check their viability, washed out and pre-constricted submaximally by $10^{-6}$ mol/l phenylephrine. Endothelium-dependent relaxation was investigated similarly to the protocol performed in perfused small arteries, e.g. concentration-response curves to acetylcholine ($10^{-9}$-$10^{-4}$ mol/l) were obtained in absence and subsequently in presence of indomethacin ($10^{-5}$ mol/l) and indomethacin+L-NMMA ($10^{-4}$ mol/l) to investigate the contribution of PGs, NO and EDHF to endothelium-mediated relaxation. In other rings, dose-response curves to phenylephrine ($10^{-9}$-$10^{-5}$ mol/l) were followed by measurements of reactivity to sodium nitroprusside ($10^{-10}$-$10^{-5}$ mol/l).

**Chemicals**

Krebs solution had a following composition (mM): 120.4 NaCl, 5.9 KCl, 2.5 CaCl$_2$, 1.2 MgSO$_4$, 25.0 NaHCO$_3$, 1.2 NaH$_2$PO$_4$, 11.5 glucose. All these compounds were purchased from Merck (Darmstadt, Germany). VAS, NS398 and SQ29548 were purchased from Cayman Chemical (Ann Harbor, MI, USA). All other drugs were obtained from Sigma-Aldrich Chemie, the Netherlands. They were dissolved either in ethanol (VAS, NS398, SQ29548) or in de-ionized water and diluted with Krebs solution. Stock solution for indomethacin was prepared in 96 mmol NaHCO$_3$.

**Statistical analysis and calculations**

Data are expressed as mean ± standard error of means (SEM). Myogenic tone, describing myogenic behaviour of an artery at a given pressure, was expressed as percent decrease in active diameter from the maximally dilated (passive) diameter determined at the same
pressure in calcium-free/EGTA solution, i.e., myogenic tone ($\%$) = $100 \times [\frac{(D_{Ca-free} - D_{Ca})}{D_{Ca-free}}]$, where D is the diameter in calcium-free ($D_{Ca-free}$) or calcium-containing ($D_{Ca}$) Krebs.

The myogenic index, describing myogenic reactivity of an artery in response to a pressure change, i.e. the slope of active pressure-diameter relationship, was calculated for every 20 mmHg pressure step ($\Delta P$) as a percentage change in corresponding active diameter $D_{Ca}$, i.e. myogenic index ($\%$/mmHg) = $100 \times [\frac{\Delta D_{Ca}/D_{Ca}}{\Delta P}]$. For each individual artery maximal myogenic tone and peak myogenic index were determined from all the pressures and pressure steps studied, respectively. Concentration-response curves to the vasoconstrictors

### Table 1. In vivo characteristics of FHL and FHH rats showing either no (7 weeks of age) or minor (12 weeks of age) renal damage.

<table>
<thead>
<tr>
<th></th>
<th>No renal damage</th>
<th>Minor renal damage</th>
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<tbody>
<tr>
<td></td>
<td>FHL</td>
<td>FHH</td>
</tr>
<tr>
<td><strong>Body weight (g)</strong></td>
<td>215 ± 3</td>
<td>211 ± 3</td>
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<tr>
<td><strong>SBP (mmHg)</strong></td>
<td>118 ± 3</td>
<td>130 ± 4*</td>
</tr>
<tr>
<td><strong>DBP (mmHg)</strong></td>
<td>76 ± 3</td>
<td>86 ± 3*</td>
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<tr>
<td><strong>Fluid intake (ml/24h)</strong></td>
<td>33 ± 5</td>
<td>40 ± 3</td>
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<tr>
<td><strong>Urine output (ml/24h)</strong></td>
<td>19 ± 2</td>
<td>23 ± 2</td>
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<tr>
<td><strong>Proteinuria (mg/24h)</strong></td>
<td>19 ± 1</td>
<td>17 ± 2</td>
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<tr>
<td><strong>Plasma creatinine (µmol/l)</strong></td>
<td>51 ± 3</td>
<td>50 ± 2</td>
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<tr>
<td><strong>Creatinine clearance (ml/min/100g body weight)</strong></td>
<td>7.4 ± 0.4</td>
<td>6.2 ± 0.2</td>
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<tr>
<td><strong>Kidney weight (g)</strong></td>
<td>1.1 ± 0.03</td>
<td>1.02 ± 0.03</td>
</tr>
<tr>
<td><strong>FGS score (%)</strong></td>
<td>1 ±1</td>
<td>1 ±1</td>
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* $p<0.01$ versus FHL of the same age  
# $p<0.01$ versus the same strain at age of 7 weeks  

SBP- systolic blood pressure, DBP- diastolic blood pressure, FGS- focal glomerulosclerosis
KCl and phenylephrine (PE) were calculated as a percentage change from baseline artery diameter and from maximal KCl response for small arteries and aorta, respectively. Concentration-response curves of the vasodilators ACh and SNP were expressed in percentage of pre-constriction to PE. The curves were characterized by the maximal relaxation ($E_{\text{max}}$) and the negative logarithm of acetylcholine molar concentration causing half-maximal relaxation ($pD_2$). Area Under ACh concentration-response curve (AUC) was determined (Sigma Plot, SPSS Inc., Chicago, IL, USA) and expressed in arbitrary units. The contribution of three endothelial mediators (PGs, NO and EDHF) to endothelial relaxation was calculated as a difference between corresponding AUCs. Full myogenic and concentration-response curves of ACh and SNP were compared by ANOVA for repeated measures followed by Bonferroni post hoc test for multiple comparisons. Group-comparison of animal and vascular parameters was performed by unpaired Student’s t-test. Differences were considered significant at p<0.05 (two-tailed).

**Results**

**Animal characteristics**

Characteristics of FHL and FHH rats in both experimental periods are given in Table 1. In 7 weeks old FHL and FHH rats, no renal damage was present, as evidenced by similar levels of proteinuria, FGS, plasma creatinine and creatinine clearance. Both SBP and DBP were marginally elevated in FHH rats as compared to FHL. Five weeks later, FHH rats developed significant proteinuria, without an increased incidence of FGS or loss of renal function. This suggests that in 12 weeks old FHH rats, mild renal damage is present. In contrast to proteinuria, blood pressure did not increase in both strains as compared to week 7. Additional animals (n=10 each strain) were followed for 26 weeks to confirm development of renal damage. At this age, hypertension, profound proteinuria and structural damage were present in FHH rats, but not in FHL rats (data not shown).

**Myogenic reactivity is selectively impaired in the renal vasculature of FHH rats prior to the development of renal end-organ damage**

At the age of 7 weeks, passive diameters of small renal arteries did not differ between FHL and FHH rats in the pressure range studied (Figure 1A). However, as evidenced by the differences in active curves (Figure 1A), renal arteries isolated from kidneys of FHH rats developed significantly lower myogenic tone as compared to FHL (Figure 2A). Consequently, young FHH demonstrated reduced maximal myogenic tone ($22 \pm 4.8 \text{ versus } 10.8 \pm 2.0 \%$, $p=0.03$) and the peak myogenic index ($-6.9 \pm 4.8 \text{ versus } 0.6 \pm 0.8 \%$/mmHg, $p=0.07$ for FHL versus FHH, respectively). In contrast to small renal arteries, active myogenic curves obtained in mesenteric arteries isolated from 7 weeks old rats did not differ between both strains (Figure 1C), demonstrating a similar level of systemic myogenic tone in FHL and FHH rats (Figure 2C). Therefore, before any renal end-organ
damage is present, myogenic response seems impaired selectively in renal vasculature of FHH rats.

**Figure 1.** Reactivity of small renal (A, B) and small mesenteric (C, D) arteries to increase of intraluminal pressure. Curves were recorded in presence (active) and in absence (passive) of extracellular calcium. Arteries were isolated from FHL and FHH rats showing either no (7 weeks of age; A, C) and or minor (12 weeks of age; B, D) renal damage.

Selective renal impairment of myogenic reactivity is more pronounced after the development of proteinuria

At 12 weeks of age, small renal arteries from FHL rats showed marked difference between active and passive myogenic curves (*Figure 1B*), developing more pronounced myogenic tone (*Figure 2B*) when compared to 7 weeks old animals (*Figure 2A*). In contrast, the level of myogenic tone in 12 weeks old FHH rats remained minimal and significantly different from FHL animals (*Figure 2B*), as reflected by markedly impaired maximal myogenic tone and peak myogenic index in FHH rats as compared to FHL (*Table 2*). In small mesenteric arteries at 12 weeks, active myogenic curves (*Figure 1D*) and myogenic tone (*Figure 2D*) development were comparable in FHL and FHH. As a result, maximal myogenic tone and
peak myogenic index in mesenteric arteries (\textit{Table 2}) did not differ between FHL and FHH rats. Therefore, selective impairment of myogenic reactivity in small renal arteries is even more pronounced after the development of renal damage.

\textbf{Figure 2.} Changes in myogenic tone of small renal (A, B) and small mesenteric (C, D) arteries in FHL and FHH rats showing either no (7 weeks of age; A, C) or minor (12 weeks of age; B, D) renal damage. * \( p<0.05 \)

To investigate whether impaired contractile ability of small arteries is confined to myogenic stimuli or a general impairment of vascular contraction, we investigated depolarization- and receptor-mediated contraction to KCl and PE, respectively. Depolarization- and receptor-mediated contractile ability in both vascular beds studied were similar between FHL and FHH rats, as evident from the characteristics of the concentration-response curves to KCl and PE shown in \textit{Table 2}. To explore whether the endothelium plays a role in the impairment of myogenic tone in renal arteries of FHH rat, we repeated the myogenic protocol after removal of the endothelium. As shown in \textit{Figure 3}, removal of the endothelium did not attenuate the differences in myogenic reactivity between FHL and FHH rats.
Table 2. Characteristics of vasoreactivity of small renal, small mesenteric arteries and aorta isolated from FHL and FHH rats at the age of 12 weeks:
parameters of response curves to potassium chloride (KCl), α-agonist phenylephrine (PE), increased intraluminal pressure (myogenic tone), endothelium-dependent vasodilator acetylcholine (ACh) and endothelium-independent vasodilator sodium nitroprusside (SNP); $E_{\text{max}}$- maximal contractile response in % of baseline vascular diameter (contractility) or in % of pre-constriction (relaxation), $pD_2$- negative logarithm of the molar concentration of agonist causing half of the maximal responses, PMI- peak myogenic tone- maximal slope of active myogenic curve (%/mmHg), * $p<0.05$

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<tr>
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<th>FHL</th>
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<tr>
<td><strong>Renal artery</strong></td>
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<tr>
<td><strong>Contractility</strong></td>
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<tr>
<td>KCl $E_{\text{max}}$ (%)</td>
<td>68 ± 4</td>
<td>71 ± 3</td>
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<tr>
<td>$pD_2$</td>
<td>1.33 ± 0.08</td>
<td>1.40 ± 0.04</td>
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<td>PE $E_{\text{max}}$ (%)</td>
<td>68 ± 4</td>
<td>70 ± 3</td>
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<td>$pD_2$</td>
<td>6.6 ± 0.1</td>
<td>6.8 ± 0.2</td>
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<tr>
<td>Myogenic $E_{\text{max}}$ (%)</td>
<td>34 ± 5</td>
<td>16 ± 2*</td>
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<tr>
<td>PMI (%/mmHg)</td>
<td>-3.2 ± 1.0</td>
<td>-0.9 ± 0.4*</td>
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<td><strong>Relaxation</strong></td>
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<tr>
<td>ACh $E_{\text{max}}$ (%)</td>
<td>66 ± 4</td>
<td>60 ± 4</td>
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<tr>
<td>$pD_2$</td>
<td>6.5 ± 0.1</td>
<td>6.8 ± 0.2</td>
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<tr>
<td>SNP $E_{\text{max}}$ (%)</td>
<td>88 ± 3</td>
<td>86 ± 3</td>
</tr>
<tr>
<td>$pD_2$</td>
<td>6.9 ± 0.2</td>
<td>6.9 ± 0.2</td>
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<tr>
<td><strong>Mesenteric artery</strong></td>
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<tr>
<td><strong>Contractility</strong></td>
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<tr>
<td>KCl $E_{\text{max}}$ (%)</td>
<td>83 ± 4</td>
<td>82 ± 3</td>
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<tr>
<td>$pD_2$</td>
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<tr>
<td>PE $E_{\text{max}}$ (%)</td>
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<td>$pD_2$</td>
<td>6.5 ± 0.1</td>
<td>6.7 ± 0.1</td>
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<tr>
<td>Myogenic $E_{\text{max}}$ (%)</td>
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<td>39 ± 4</td>
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<tr>
<td>PMI (%/mmHg)</td>
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<td><strong>Relaxation</strong></td>
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<tr>
<td>ACh $E_{\text{max}}$ (%)</td>
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<td>97 ± 1*</td>
</tr>
<tr>
<td>$pD_2$</td>
<td>7.2 ± 0.1</td>
<td>6.6 ± 0.1*</td>
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<td>SNP $E_{\text{max}}$ (%)</td>
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<td>97 ± 2</td>
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<td>$pD_2$</td>
<td>7.3 ± 0.2</td>
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<tr>
<td><strong>Aorta</strong></td>
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<tr>
<td><strong>Contractility</strong></td>
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<tr>
<td>PE $E_{\text{max}}$ (% of KCl)</td>
<td>75 ± 5</td>
<td>78 ± 5</td>
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<td>$pD_2$ (% of KCl)</td>
<td>6.7 ± 0.1</td>
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<td><strong>Relaxation</strong></td>
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<td>ACh $E_{\text{max}}$ (%)</td>
<td>56 ± 4</td>
<td>57 ± 8</td>
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<tr>
<td>$pD_2$</td>
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</tr>
<tr>
<td>SNP $E_{\text{max}}$ (%)</td>
<td>98 ± 1</td>
<td>100 ± 1</td>
</tr>
<tr>
<td>$pD_2$</td>
<td>7.9 ± 0.1</td>
<td>7.7 ± 0.2</td>
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Renal and systemic endothelial dysfunction in FHH rats prior to the development of renal end-organ damage

Acetylcholine (ACh) relaxed small renal arteries isolated from 7 weeks old FHL rats, resulting in monophasic concentration-response curve (Figure 4A). In FHH rats however, a biphasic curve was observed as higher concentrations of ACh induced a contractile response (Figure 4A). In contrast to renal artery, ACh incubation resulted in monophasic concentration-response curve in both FHL and FHH rats in small mesenteric arteries, although the curve was shifted to the right in FHH rats (Figure 4C). Unlike in small arteries, no difference between ACh-induced relaxations of the two strains was observed in aortic rings (Figure 4E). All observed alterations specifically indicated the presence of endothelial dysfunction, since no differences were found in the reactivity to endothelium-independent vasodilator SNP in any vascular bed between FHL and FHH rats (Table 2).

Progression of systemic endothelial dysfunction in FHH rats after the development of proteinuria

At the age of 12 weeks, animals showed a similar pattern of endothelial dysfunction in the investigated vascular beds as observed at the age of 7 weeks. In 12 weeks old animals, endothelial dysfunction was present in small renal (Figure 4B) and mesenteric artery (Figure 4D), but not in aorta (Figure 4F). In small mesenteric artery, endothelial dysfunction in FHH was aggravated when compared to 7 weeks old rats, as evidenced by a further shift of the response curve to the right (Figure 4D). SNP-induced relaxation remained unchanged in all vascular beds.
Figure 4. Endothelial dysfunction in various vascular beds of FH rats. Endothelium-mediated vasodilation to acetylcholine measured in small renal arteries (A, B), small mesenteric arteries (C, D) and aorta (E, F) isolated from FHL and FHH rats showing either no (7 weeks of age; A, C, E) or minor (12 weeks of age; B, D, F) renal damage. * p<0.05
Figure 5. Heterogeneous mechanisms of endothelial dysfunction between 12 weeks old FHL (A, C, E) and FHH (B, D, F) rats in small renal (A, B), small mesenteric arteries (C, D), and aorta (E, F). Endothelium-mediated vasodilation to acetylcholine in absence (total) of any inhibitors and in the presence of indomethacin (indo, $10^{-5}$ mol/l), combination of indomethacin and L-NMMA ($10^{-4}$ mol/l) and eventually in additional presence of charybdotoxin (chtx, $10^{-7}$ mol/l) and apamin (apa, $3 \times 10^{-7}$ mol/l). * $p<0.05$ indo versus indo+L-NMMA, # $p<0.05$ indo+L-NMMA+chtx+apa versus all other curves, & $p<0.05$ indo versus total.
Heterogeneous mechanisms underlying endothelial dysfunction in FHH rats

To explore the mechanisms responsible for the endothelial dysfunction in studied vascular beds, we constructed ACh concentration response-curve in presence of the inhibitors of endothelium-derived vasodilatory mediators in both 7 and 12 weeks old animals. Since similar curves were obtained at both time points, for reasons of clarity only data from week 12 are presented in Figure 5. Indomethacin, an inhibitor of cyclooxygenase (COX), completely reversed endothelium-dependent contractions associated with higher doses of ACh in small renal arteries of FHH rats (Figure 5B), while it had no significant effect in FHL rats (Figure 5A). Additional blockade of NO by L-NMMA resulted in significant attenuation of ACh-vasodilation in renal arteries of FHH rats (Figure 5B), while affecting the relaxation to a lesser extent in FHL animals (Figure 5A). The remaining ACh-relaxation was completely blocked by the combined application of potassium channels inhibitors charybdotoxin and apamin suggesting this response is mediated by EDHF (Figure 5A, B). Indomethacin did not affect the endothelial dilation in either FHL or FHH rats in small mesenteric artery (Figure 5C, D). Additional block of NO led to a small right-shift of the curve in FHL (Figure 5C), with even less pronounced effect in FHH (Figure 5D). Similar to renal vessels, the remaining relaxation was completely inhibited by charybdotoxin and apamin (Figure 5C, D).

In aorta, the ACh-induced response was not altered in the presence of indomethacin, whereas it was almost completely attenuated by NO blockade. No differences were observed between FHL and FHH rats (Figure 5E, F).

Based on these observations, the contribution of the principal endothelial mediators (PGs, NO, EDHF) to endothelial function was calculated. The release of constrictive PGs seems responsible for endothelial dysfunction in small renal arteries of FHH prior to the development of renal damage, as indicated by negative value of PGs contribution in Figure 6A. At the same time, PGs do not play any role in endothelial dysfunction in the mesenteric artery, in which rather a reduction in EDHF is found (Figure 6C). In aorta, no changes in endothelial mediators could be detected (Figure 6E).

After the development of renal damage, endothelial dysfunction persists in FHH rats with similar mechanisms involved. Renal arteries showed comparable production of contractile PGs (Figure 6B) as observed prior to the development of renal damage (Figure 6A). In contrast, mesenteric arteries displayed even more pronounced loss of EDHF-mediated relaxation compared to 7 weeks old FHH rats (Figure 6D). No significant changes were observed in the responses of aorta (Figure 6F). Additionally, small renal arteries of FHH rats with renal damage rely relatively more on NO-mediated vasodilation than FHL rats (Figure 6B).
Figure 6. Heterogeneous contribution of endothelial mediators prostaglandins (PGs), nitric oxide (NO) and endothelium-dependent hyperpolarizing factor (EDHF) to endothelium-dependent relaxation shown in arbitrary units of Area Under acetylcholine concentration-response curve (AUC) in small renal (A, B), small mesenteric arteries (C, D) and aorta (E, F) of FHL and FHH rats showing either no (7 weeks of age, A, C, E) or minor (12 weeks of age; B, D, F) renal damage. *p<0.05
Mechanisms underlying endothelium-mediated contractions in renal arteries of FHH rats
To identify the mechanisms underlying ACh-mediated indomethacin-sensitive contractile response in renal arteries of FHH rats, additional experiments were performed in 12 week old rats. As shown in Figure 7, endothelium-dependent contractions to ACh are reversed to relaxations in the presence of COX-1 inhibitors, but unaffected by COX-2 inhibitors. Furthermore, the contractions were blunted by a TXA<sub>2</sub>/PGH<sub>2</sub> receptor antagonist and by superoxide dismutase, identifying the target receptor of the COX1-derived prostanoids and suggesting involvement of reactive oxygen species in this response, respectively.

**Figure 7.** The effect of cyclooxygenase pathway inhibitors on prostanoid-mediated endothelium-dependent contractions in small renal arteries of 12 weeks old FHH rats. VAS- valeryl salicylate (10<sup>-4</sup> mol/l)- COX-1 selective inhibitor, NS398 (10<sup>-6</sup> mol/l)- COX-2 selective inhibitor, SQ29548 (10<sup>-6</sup> mol/l)- TXA<sub>2</sub>/PGH<sub>2</sub> receptor antagonist, SOD-superoxide dismutase (50 U/ml). * p<0.05 NS398 versus all other inhibitors.

**Discussion**

The present study reports a severely impaired myogenic contractility of small renal arteries in an inbred rat model of spontaneous renal disease (FHH) when compared to its disease-resistant counterpart (FHL). Moreover, FHH rats displayed a COX-1-dependent endothelial dysfunction. These renal vascular changes were detected prior to the manifestation of renal damage, thus being potentially involved in the increased susceptibility of FHH rats to renal injury. Furthermore, these alterations were selectively observed in small renal, but not in small mesenteric arteries, in which only endothelial dysfunction due to impaired EDHF-mediated vasodilation was found. Finally, impaired vascular reactivity was further
aggravated during the course of spontaneous renal damage. Therefore, heterogeneous vascular dysfunction is present in various vascular beds in animal models of spontaneous renal disease prior to the development of end-organ damage.

A progressive pattern of spontaneous hypertension-associated renal disease in young Fawn-Hooded rats as observed in our study is in agreement with previous reports\textsuperscript{19,20}. Seven weeks old FHH rats showed minor elevation in blood pressure as compared to age-matched FHL counterparts. However, blood pressure of both strains remained in the normotensive range at that age. At this time point, markers of renal damage did not differ between experimental strains. Although blood pressure did not increase further in FHH rats over the following 5 weeks, a marked elevation in urinary protein excretion was observed. Renal damage progresses rapidly in FHH strain and eventually leads to end-stage renal failure-related death before the age of 70 weeks\textsuperscript{21}, whereas FHL rats do not develop early hypertension and renal lesions\textsuperscript{14}. Therefore, FHH rats studied at an early age provide a useful model to investigate the role of vascular changes in the development of spontaneous renal damage and systemic vascular complications.

Our observation of marked attenuation of myogenic reactivity in small renal arteries of FHH rats as compared to FHL strain is in agreement with observations of van Dokkum et al.\textsuperscript{16}, who reported significantly lower myogenic constriction in renal interlobular artery of proteinuric FHH rats. We extend these data by showing that the difference in myogenic reactivity between FHL and FHH is detected prior to development of proteinuria. In addition, we found that myogenic reactivity increases with age in FHL, whereas this increase is absent in FHH rats. This observation suggests that the myogenic mechanism in FHH rats fails to adapt to increased hemodynamic load during the progression of hypertension or associated renal disease. Altered myogenic reactivity may result in an impaired autoregulatory ability of the kidney, leading to elevated glomerular capillary pressure, subsequent hyperfiltration and progressive proteinuria. Although we were not able to detect hyperfiltration, as calculated creatinine clearance was similar in both studied groups, Simons et al. demonstrated an elevation of intraglomerular capillary pressure in FHH rats at the age of 7 weeks\textsuperscript{15}, i.e. before the development of proteinuria. Furthermore, an attenuated autoregulation of renal blood flow has been reported in 12 weeks old FHH rats as compared to FHL\textsuperscript{22}. Recently, it has been shown that autoregulation is restored and proteinuria reduced by the transfer of a specific region of chromosome 1 from Brown-Norway rats to FHH\textsuperscript{23}, indicating that altered autoregulation may be responsible for the genetic susceptibility of FHH strain to renal disease. Collectively, these data indicate that impaired myogenic reactivity may represent the mechanism rendering a given animal strain susceptible to renal injury.

Interestingly, the impairment of myogenic constriction is specific for the renal vasculature, since no differences in myogenic reactivity between FHH and FHL rats were found in the systemic resistance arteries. This suggests that myogenic response of various vascular beds is heterogeneously affected in FHH rats and that differential mechanisms underlie myogenic reactivity in renal and mesenteric arteries. However, only few studies reported
heterogeneity of myogenic mechanisms among vascular beds \textsuperscript{24} and differential mechanisms have not been characterized yet. Generally, myogenic constriction is a result of intrinsic reactivity of smooth muscle cells associated with membrane depolarization and increase in intracellular calcium levels. Several mechanosensitive ion channels and integrins may be involved in a stretch-induced mechanotransduction, whereas other factors have been implicated in the myogenic signal transduction, including activation of calmodulin/ myosin light chain kinase pathway, PKC, MAP kinases, cytochrome P450-derived metabolites of arachidonic acid (20-HETE) and several potassium channels \textsuperscript{25}. Altered activity of any of these components may underlie the impairment of renal myogenic constriction FHH rats, although several of these mechanisms participate also in receptor- or depolarization-mediated contraction. Since these contractions were intact, the affected mechanism in FHH seems rather specific for the stretch-induced reactivity, such as mechanotransduction. Moreover, the defect is independent of basal reactivity of endothelium, since impairment of myogenic constriction in FHH persisted after removal of the endothelium. Thus, reduced myogenic reactivity in small renal arteries may explain the susceptibility of FHH rats to renal damage. In addition, its selective impairment in renal arteries indicates that different mechanisms are involved in the generation of myogenic responses across different vascular beds.

We have previously shown that variation in renal endothelial function of healthy rats from an outbred Wistar strain predicts their susceptibility to the development of renal end-organ damage after renal mass reduction \textsuperscript{9} and combined unilateral nephrectomy and myocardial infarction \textsuperscript{26}. Healthy individuals with pronounced endothelium-dependent relaxation developed less severe renal damage. The current finding that endothelial function in FHH is already impaired at an early age prior to the development of renal damage is in agreement with these observations in outbred strains and suggests that endothelium actively participates in the susceptibility to end-organ damage.

The primary mechanism underlying endothelial dysfunction in renal arteries of FHH rats is COX-1-mediated endothelium-dependent contraction, in which the production of vasoconstrictive endoperoxides and/or thromboxanes and superoxide radical were involved. In a previous report from our lab, we showed that pronounced production of endothelial contractile prostanoids in healthy rats was associated with the excessive renal damage induced by subsequent renal mass reduction. Interestingly, early hyperfiltration in FHH rats was reported to coincide with an excessive urinary excretion of contractile prostaglandins \textsuperscript{27}. These observations may suggest the role of prostaglandins in the development of spontaneous renal injury. However, spontaneously hypertensive rat (SHR), a strain relatively resistant to the development of renal injury also displays COX-mediated renal endothelial dysfunction at an early, pre-hypertensive stage \textsuperscript{28}. Therefore alternatively, renal vasoconstrictive prostaglandins may play the critical role in the development of spontaneous hypertension \textsuperscript{29}. Interestingly, while endothelium-dependent contraction is confined exclusively to renal vasculature in FHH, it is found also in systemic resistance arteries and aorta in SHR \textsuperscript{30-32}. However, the implications of this observation remain unclear.
and further research is needed to clarify the role of early renal endothelium-dependent contractions in the pathophysiology of hypertension-associated renal damage in FHH rats. Reduced bioavailability of nitric oxide (NO) is considered to be a key mechanism for renal endothelial dysfunction in various experimental models of hypertension and renal damage\textsuperscript{33,34}. We previously reported that pronounced renal NO-mediated vasodilation is associated with protection against the development of renal damage in several models of renal injury\textsuperscript{9,10}. However, in the present study, no difference was found in NO-mediated vasodilation between FHL and FHH rats prior to the development of renal damage. Moreover, in proteinuric FHH animals, the NO-dependent vasodilation is increased rather than reduced, suggesting that NO bioactivity is modified during the course of renal disease. Likewise, increased NO activity has been described in early stages of diabetic nephropathy and has been linked to hyperfiltration\textsuperscript{35,36}. One might speculate about a similar mechanism in FHH rats, leading to elevated glomerular pressure and the development of proteinuria. Alternatively, increased NO release might represent a compensatory mechanism in response to the production of COX-derived reactive oxygen species. Interestingly, increased constitutive nitric oxide synthase-1 (NOS-1) expression has been described in macula densa of young FHH rats\textsuperscript{37}, however it is unclear whether NO signaling in preglomerular arteries is affected. NO-related changes in FHH rats seem to be limited to the renal vasculature and was not observed in systemic vascular beds, even though acetylcholine-induced relaxation almost entirely relies on NO in the aorta. Taken together, these data indicate that reduced NO-mediated relaxation does not seem to represent a major mechanism of early vascular dysfunction in spontaneous renal disease.

In small arteries, such as renal or mesenteric artery, the majority of endothelial relaxation under physiological condition is mediated by EDHF\textsuperscript{9,38,39}. Interestingly, this mechanism was intact in small renal, but impaired in mesenteric resistance artery of FHH rats at both time points investigated. This heterogeneity might be explained by the variable identity of EDHF in different vascular beds\textsuperscript{40-44}. Nevertheless, it is well established that in all vascular beds activation of the endothelial calcium-regulated potassium K\textsubscript{Ca} channels is critical for EDHF-response, as evidenced in our study by the complete blockade of EDHF-mediated vasodilation in the different arteries by charybdoxtoxine and apamin, inhibitors of the large/intermediate (BK\textsubscript{Ca}/IK\textsubscript{Ca}) and small conductance (SK\textsubscript{Ca}) channels, respectively. Selectively altered EDHF-mediated relaxation in systemic arteries appears before the development of proteinuria, suggesting that generalized endothelial dysfunction may precede renal injury. EDHF reduction in resistance arteries may possibly be related to the minor blood pressure increase in FHH rats. However, it seems unlikely, because during the course of the study EDHF further progressed with proteinuria increase, despite unchanged blood pressure. Moreover, impaired EDHF-mediated relaxation observed in various vascular beds following experimental renal disease induced by renal mass reduction\textsuperscript{45,46} is independent from blood pressure increase. Changes in the expression of endothelial SK\textsubscript{Ca} and IK\textsubscript{Ca} channels underlying the EDHF loss in this model\textsuperscript{45}, might be also involved in endothelial dysfunction in FHH. Collectively, impaired EDHF-mediated relaxation
represents the earliest systemic vascular alterations in spontaneous hypertension-associated renal disease.

In conclusion, the present study shows that vascular dysfunction in both small renal and systemic arteries precedes renal damage in FHH rats, a model of spontaneous hypertension-associated damage. This study confirms the early presence of vascular impairment throughout various vascular beds in individuals susceptible to renal disease, however it reports marked heterogeneity in affected vasomotor mechanisms between small renal, resistance and conduit arteries. Future research should be warranted to define the role of endothelial and smooth muscle reactivity in the development of renal end-organ damage.
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