Baseline Intrarenal Artery Function Predicts Proteinuria in Obese Rats Prone to Develop Diabetes

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(Submitted)
Abstract

Zucker-diabetic-fatty rats (ZDF) are prone to develop diabetes-mellitus (DM) and nephropathy associated herewith. Here we studied the predictive-value of renal-artery function at pre-diabetic state in relation to future renal-damage during DM-development. Young-ZDF were subjected to unilateral-nephrectomy (UNx) (n=10), the endothelium-dependent-relaxation (EDR) and myogenic-constriction (MC) determined in intrarenal-arteries obtained from the individual's extirpated kidney and correlated with proteinuria-increase 10-weeks thereafter as an indices of renal damage. Except for body-weight, metabolic parameters of young-ZDF were within normal range, indicating they were at the early onset of DM at the start of the study. UNx induced an initial steep-rise in proteinuria followed by a gradual-increase thereafter when ZDF developed signs of DM but with normotensive blood-pressure. The overall-proteinuria-increase following UNx and DM correlated with baseline EDR (r=-0.856, p=0.002) and MC (r=0.789, p=0.020). The findings demonstrate that baseline intrarenal-artery function predicted future renal-injury in obese subjects prone to develop DM. The smaller EDR predicting higher proteinuria-increase suggests that reinforcement of endothelial function is a relevant target to prevent renal-damage in DM. In contrast, the larger MC predicting higher proteinuria-increase is opposite from findings in hypertensive models of renal-damage. This suggests for further investigation of the predictive value of MC in DM with(-out) hypertension.
1. Introduction

Despite displaying similar risk profiles, the development of chronic kidney disease (CKD) varies considerably among patients with conditions such as diabetes mellitus (DM), dyslipidemia and hypertension (HT) [1]. The high variability of development of renal damage between individuals also shows racial differences and genetic components [2-5]. Although the underlying mechanisms of this individual susceptibility to CKD are still largely unknown, a patent endothelial function as a powerful defense mechanism against organ injury has been implied [6,7]. Consistent with that we showed that baseline endothelial dilatory function of intrarenal arteries may predict an individual’s susceptibility to CKD in a proof of principle study. In that study a smaller endothelium-dependent relaxation (EDR) measured at baseline in normal healthy rats predicted a higher degree of experimentally induced renal damage after 5/6 renal mass reduction [8]. However, whether baseline EDR also predicts CKD in natural or clinical courses of renal injury such as during DM development has not been studied previously.

In addition to endothelial function and (local) neurohumoral mechanisms, myogenic responsiveness of small arteries also plays an important role in the organ’s microvascular function [9]. Intact myogenic constriction (MC) - i.e. the intrinsic ability of small vessels to constrict in response to increased intraluminal pressure - is believed to protect from exposure to large pressure and flow fluctuations and the potential damage hereof in end-organs such as the eye, brain and kidney [9,10]. In Fawn-Hooded Hypertensive rats, loss of MC of small intrarenal arteries precedes hypertensive renal damage [11,12]. In normal rats, baseline MC predicted hypertensive renal damage after experimental 5/6 renal mass reduction [13]. However, whether baseline MC of intrarenal arteries may also have a predictive value for renal damage during DM development is unknown.

It is hypothesized that the small intrarenal artery function plays an important role in the susceptibility and progression of renal injury in DM [9,14]. To study this we subjected young Zucker diabetic fatty rats (ZDF) rats to unilateral nephrectomy (UNx) when 7 weeks of age and still at the early onset of DM. Baseline renal artery function of the individual's extirpated kidney was assessed as EDR and MC and correlated with subsequent increases in proteinuria as an indices of renal damage development during 10 weeks thereafter.

2. Materials and Methods
2.1. Animals and study design. The protocols for animal care and use were 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 (Permit Number: DEC6032B). Studies were conducted using male obese ZDF (fa/fa) rats obtained from Charles River (France). Animals were housed group-wise in standard cages and maintained on a 12:12-hour light:dark cycle with free access to food (Purina LabDiet Formulab 5008) and normal tap drinking water throughout the study. After an one-week acclimatization period when animals were at 7 weeks of age, a blood sample was drawn from the tail vein and animals were put in metabolic cages for 24 hours for baseline measurements before they underwent UNx. For this, animals were anesthetized with 2.5% isoflurane in O_2, the right kidney removed and the interlobular renal artery collected for vascular studies (ZDF-UNx, n=10). Postoperatively, all animals received buprenorphin (Temgesic®; 0.01 mg/kg) subcutaneously for analgesic purposes. Animals were followed until 17 weeks of age and 24h urinary protein excretion was assessed every 2 weeks. To control for the effect of UNx on the development of proteinuria in a setting DM development, another group of age-matched ZDF rats was sham operated and concomitantly followed for proteinuria (ZDF-sham, n=8). To further control for the effect of UNx per se on the development of proteinuria in the absence of DM, two additional groups of age-matched Zucker lean control rats (+/?) were either sham- or UNx-operated and concomitantly followed for 24h urinary protein excretion (lean-sham, n=8; lean-UNx, n=7).

Ten weeks after surgery, ZDF-UNx rats were anesthetized (2.5% isoflurane in O_2) and hemodynamic parameters measured by an invasive pressure transducer catheter (Micro-Tip 3-French; Millar Instruments Inc., Houston, Tex., USA) inserted through the right carotid artery into the aortic root. After hemodynamic measurements, the ZDF-UNx animals were terminated by exsanguination and the kidney removed for analyses of glomerulosclerosis.

2.2. Vascular measurements of baseline intrarenal artery function. Immediately after UNx, the nephrectomized kidney was weighed and transferred to the vascular laboratory. Small intrarenal arteries with an intraluminal diameter of 329±19µm were cleaned from perivascular tissue and used for measuring endothelial function and myogenic tone, as described previously [8,11,13]. For MC arteries were mounted in a perfusion pressurized setup (Living System Instrumentation, Burlington, VT, USA). In short, artery segments were cannulated on glass micropipettes in the vessel chamber that was filled and continuously recirculated with warmed (37°C) and oxygenated (5% CO_2 in O_2) Krebs
solution with a pH of 7.4. An inverted light microscope attached to a video camera and video dimension analyzer was used to evaluate the lumen diameter. Vessels were allowed to equilibrate for 30 min with 60 mmHg intraluminal pressure. Arteries were checked for smooth muscle viability and the endothelium denuded by means of a single high dose of phenylephrine (PE, 1 µmol/L). Subsequently, removal of the endothelium was confirmed by an absence of a response to methacholine (MCh; 20 µmol/L). Following a wash out, intraluminal pressure was equilibrated to 20 mmHg for 3 min. MC was assessed by a stepwise increase (20 mmHg) of the pressure up to 140 mmHg. Each pressure step was maintained for 3 minutes to reach a stable contraction. To obtain passive pressure curves the procedure was repeated but in the absence of calcium (calcium-free Krebs solution supplemented with ethyleneglycol-bis-(b-aminoethylether)-tetraacetic acid (EGTA, 2 mmol/L).

For assessment of endothelial function arteries were cut into 2-3 mm segments and mounted in a myograph setup (DMT model 610M, Denmark) filled with warm Krebs solution. As described previously [15], optimal wall tension was calculated as the vessel segment was stretched by stepwise increasing the distance between clamps in steps of 10 µm until the transmural pressure exceeded 100 mmHg. The internal circumference and corresponding wall tension were fitted on an exponential curve for determination of L100 (calculated diameter of the vessel at 100 mmHg). After calculation of the optimal wall tension, arteries were allowed to equilibrate for 30 minutes at 0.9 L100 before being pre-constricted with 0.3 µmol/L PE. Pre-constricted vessels were studied for EDR by applying cumulative doses of MCh (1 nmol/L - 10 µmol/L). After addition of the last dose of MCh, a single high dose of sodium nitroprusside (SNP, 1 mmol/L) was given to test for maximal endothelium independent dilation. EDR and MC were successfully determined for 10/10 and 8/10 ZDF-UNx rats, respectively. To confirm that baseline renal artery endothelial function and myogenic tone in young ZDF rats was within normal range, comparisons were made to 7 week old lean control rats.

2.3. Clinical chemistry. Blood glucose and cholesterol were determined using Accu-Chek Aviva and AccuTrend (both Roche Diagnostics, Almere, Netherlands), and Hb1Ac using DCA Vantage (Siemens Healthcare Diagnostics Inc., Deerfield, IL) analyzers, respectively. Plasma and urine creatinine were measured by means of a photometric assay with the Jaffé method without deproteinization (DiaSys Diagnostics Systems, Holzheim, Germany).
Urinary protein excretion was measured in 24h urine collections by trichloroacetic acid precipitation (Nephelometer analyzer II; Dade Behring, Marburg, Germany).

2.4. Renal histology. Coronal tissue slices through the mid portion of the kidney were fixed in 4% formaldehyde and embedded in paraffin, after which sections (4 μm) were stained with periodic acid-Schiff (PAS). Subsequently, glomeruli were semi-quantitatively scored for focal glomerulosclerosis (FGS) by light microscopy on a scale of 1 to 4, as described previously [16]. FGS was scored positive when mesangial expansion, mesangial cellularity, adhesion formation, and capillary obliteration was present in one segment. If 25% of glomerulus was affected, a score of 1 was adjudged, 50% was scored as 2, 75% as 3, and 100% as 4. The ultimate score is then obtained by multiplying the degree of change by the percentage of glomeruli with the same degree of injury and adding these scores. A total of 50 glomeruli per kidney were scored moving from cortex to medulla and the average value per kidney (i.e. per animal) calculated. The scoring was performed by two researchers in a blinded fashion.

2.5. Solutions and drugs. The composition of Krebs solution was (in mmol/L): NaCl (120.4), KCl (5.9), CaCl₂ (2.5), MgCl₂ (1.2), NaH₂PO₄ (1.2), glucose (11.5), NaHCO₃ (25.0) at pH 7.4. Compounds for Krebs solution were obtained from Merck (Darmstadt, Germany) and all other drugs from Sigma-Aldrich (St. Louis, MO, USA).

2.6. Calculations and data analysis. Data are presented as mean ± SEM; n values represent the number of investigated rats. IBM SPSS Statistics for Windows, Version 20.0 (Armonk, NY: IBM Corp.) was used for statistical analysis. In vascular studies, MC 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., MC (%) = 100 [(Dₐₙₙ – Dₙₙ)/Dₙₙ], where D is the diameter in calcium free (Dₐₙₙ) or calcium-containing (Dₙₙ) Krebs. EDR responses to MCh were expressed as a percentage of pre-constiction. Full pressure-myogenic response curves (in case of MC), concentration-relaxation curves (in case of EDR), and time-course curves (in case of proteinuria) were compared using repeated measures ANOVA. In addition to that the Area Under Curve (AUC, in arbitrary units) was determined for each individual MC and EDR curve and used for correlation and univariate linear regression analysis with parameters of renal damage. Unless stated otherwise, comparisons were performed using Student's paired or unpaired t-test, as appropriate; p<0.05 values were considered statistically significant.
3. Results

3.1. Physical and biochemical parameters of obese ZDF rats. The data in Table 1 show that 17 week old ZDF rats had developed clear features of DM as blood values for plasma glucose, HbA1c, cholesterol and plasma creatinine (P\. Cr) all were significantly increased compared to their respective values at 7 weeks of age. Renal parameters, such as urine production, creatinine clearance (C\. Cr), protein excretion and FGS score, additionally demonstrated the development of renal damage once at 17 weeks of age. In contrast, at the start of the study when the animals were 7 weeks of age the metabolic parameters were within the normal range in ZDF and similar to that of age-matched lean control rats (8.3±0.4 mmol/L for glucose, 3.06±0.06 % for HbA1c, 2.01±0.17 mmol/L for cholesterol), except for increased body weight (269±5 and 205±5 g respectively, p<0.001). Other values for 7 week old ZDF and lean controls were: respectively 17.8±0.7 and 19.3±0.7 µmol/L for P\. Cr, 6.097±0.250 and 5.640±0.235 mg/g for kidney-to-body weight, and 12.7±6.4 and 6.6±0.6 mg/24h for urinary protein excretion (p=ns for all). The data confirm that at the moment UNx was performed the ZDF rats were obese and at the early onset of DM.

Table 1. Physical and biochemical parameters of obese ZDF rats.

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<thead>
<tr>
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<th>ZDF rats (n=10)</th>
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<tr>
<td></td>
<td>Baseline</td>
</tr>
<tr>
<td></td>
<td>(at 7 weeks of age)</td>
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<tr>
<td>Body weight (g)</td>
<td>269±5</td>
</tr>
<tr>
<td>Right kidney weight (mg)</td>
<td>1629±76</td>
</tr>
<tr>
<td>Left kidney weight (mg)</td>
<td>-</td>
</tr>
<tr>
<td>Hemodynamic parameters</td>
<td></td>
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<tr>
<td>Aortic HR (b.p.m.)</td>
<td>-</td>
</tr>
<tr>
<td>Aortic SBP (mmHg)</td>
<td>-</td>
</tr>
<tr>
<td>Aortic DBP (mmHg)</td>
<td>-</td>
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<tr>
<td>Blood parameters</td>
<td></td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>9.9±1.1</td>
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<tr>
<td>HbA1c (%)</td>
<td>3.56±0.08</td>
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<tr>
<td>Cholesterol (mmol/L)</td>
<td>2.95±0.19</td>
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</table>
Chapter 2

<table>
<thead>
<tr>
<th>P&lt;sub&gt;Cr&lt;/sub&gt; (µmol/L)</th>
<th>17.8±0.7</th>
<th>32.3±0.8***</th>
</tr>
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</table>

**Renal parameters**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value 1</th>
<th>Value 2</th>
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</thead>
<tbody>
<tr>
<td>Urine production (ml/24h)</td>
<td>8.6±1.4</td>
<td>24.4±2.3***</td>
</tr>
<tr>
<td>C&lt;sub&gt;Cr&lt;/sub&gt; (ml/min/100g)</td>
<td>0.68±0.14</td>
<td>0.38±0.06**</td>
</tr>
<tr>
<td>Protein excretion (mg/24h)</td>
<td>12.7±6.4</td>
<td>188.9±24.6***</td>
</tr>
<tr>
<td>FGS (%)</td>
<td>-</td>
<td>50.3±5.9</td>
</tr>
</tbody>
</table>

Abbreviations: UNx, unilateral nephrectomy; HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; P<sub>Cr</sub>, plasma creatinine; C<sub>Cr</sub>, creatinine clearance; FGS, focal glomerulosclerosis. Data are mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 (paired t-test).

3.2. **Inter-individual variation in baseline intrarenal artery function in young ZDF rats.**

Baseline EDR and MC was determined in intrarenal arteries obtained from the extirpated kidney at UNx. The mean concentration/pressure response curves for EDR and MC are presented in Figure 1A/B respectively, and demonstrate that at the group level small renal artery function in young obese ZDF was similar to that of age-matched lean controls. EDR and MC was also calculated as the Area Under Curve (AUC), presented for individual ZDF rats in Figure 1C. The data demonstrate normal EDR and MC in young obese ZDF with considerable inter-individual variation.
Figure 1. Baseline intrarenal artery function in young obese ZDF rats. Endothelium-dependent relaxation (EDR, panel A) and myogenic constriction (MC, panel B) in the group of young obese ZDF rats at the start of the study (open triangles) was not different from that in age-matched lean controls (closed circles); data are mean ± SEM. Panel C shows the variation in baseline EDR (n=10, left y-axis) and MC (n=8, right y-axis) of individual ZDF rats calculated as the area under the curve (AUC, arbitrary units); hence AUC was used in regression analysis of EDR and MC (see methods).
3.3. Development of proteinuria in obese ZDF rats following UNx. In the follow-up period after the nephrectomy, ZDF rats developed progressive proteinuria (Figure 2A). Interestingly, development of proteinuria in ZDF-UNx rats seemed to consist of 2 phases; an early steep rise shortly after UNx (i.e. within 2 weeks) followed by a more gradual increase over time thereafter. To discriminate between the apparently two phases of proteinuria increase, we also calculated the change in proteinuria between weeks 0 and 2 (early rise), weeks 2 to 10 (gradual rise) and the overall rise in proteinuria (weeks 0 to 10) (Figure 2B). The early steep rise in proteinuria was present in ZDF-UNx only. The gradual rise in proteinuria was present both in ZDF-UNx and ZDF-sham, albeit lower in the former, while the overall rise in proteinuria was highest in ZDF-UNx. Lean-sham and lean-UNx controls did not develop proteinuria.
Figure 2. Augmented increase in proteinuria in young obese ZDF following UNx. Starting at 7 weeks of age, 24h urinary protein excretion was determined prior and after unilateral nephrectomy (UNx) or sham-surgery in Zucker diabetic fatty (ZDF) and age-matched lean control rats for 10 weeks. Panel A shows the actual time course of urinary protein excretion measured every 2-weeks for all groups. In addition to that the net-increase in urinary protein excretion was calculated from 0-to-2 (early), 2-10 (gradual), and 0-10 (overall) weeks after surgery, as shown in panel B. Hence, a marked early rise in proteinuria was present in ZDF-UNx rats only while the gradual increase was more similar between ZDF-sham and -UNx rats; consequently the overall increase in proteinuria was highest in ZDF-UNx rats. Data are mean ± SEM. ***p<0.001 ZDF-sham vs Lean-sham; ###p<0.001 ZDF-UNx vs Lean UNx; significant differences between UNx versus sham are as indicated.
3.4. **Baseline intrarenal artery function predicting future proteinuria.** To investigate whether intrarenal artery function in young ZDF rats predicted for future renal damage development, we plotted EDR and MC against the early-, gradual- and overall-rise in proteinuria in ZDF-UNx. Baseline EDR inversely correlated with the overall rise in proteinuria (Figure 3A). The inverse trend was also seen with the early- and gradual-rise in proteinuria but these did not reach statistical significance. MC positively correlated with the gradual- and overall-, but not the early-rise in proteinuria (Figure 3B). Hence, as measurement of EDR and MC of small renal arteries requires a nephrectomy, it was technically impossible to measure EDR and MC in the ZDF-sham and lean-sham groups. Further, lean-UNx do not develop proteinuria and were therefore also excluded from this analysis.

![Graph A: Early rise (0-2 weeks), Gradual rise (2-10 weeks), Overall rise (0-10 weeks)]

![Graph B: Increase in proteinuria (mg/24h) vs baseline EDR (AUC, arbitrary units)]

![Graph B: Increase in proteinuria (mg/24h) vs baseline MC (AUC, arbitrary units)]
Figure 3. Baseline intrarenal artery function in young obese ZDF predicts future increase in proteinuria. Shown are scatterplots of renal artery endothelium-dependent relaxation (EDR, panel A) and myogenic constriction (MC, panel B) in young obese ZDF rats at the early onset of diabetes mellitus with increases in proteinuria following uninephrectomy (UNx). EDR and MC are presented as AUC (in arbitrary units). The early, gradual and overall increase in proteinuria was calculated as the difference from 0-2 (left panels), 2-10 (middle panels) and 0-10 (right panels) weeks after UNx at the start of the study. Fitted lines show the result of linear regression analysis with r- and p-values as indicated.

3.5. Other baseline factors predicting future renal damage. In addition to EDR and MC, baseline HbA1c inversely correlated with MC (Figure 4A) and also showed a significance with the overall rise in proteinuria in univariate regression analysis (Table 2). Because of that we additionally performed a stepwise multivariate linear regression analysis with baseline EDR, MC and HbA1c being the factors included in the model (F-entry=0.05, F-removal=0.10) to predict the overall rise in proteinuria. Two possible models were generated; model 1: $r^2=0.732$ (p=0.007) with $B=505.1\pm82.7$ for constant (p<0.001) and $B=-2.8\pm0.7$ for EDR with $\beta=-0.856$ (p=0.007), and model 2: $r^2=0.887$ (p=0.047) with $B=287.0\pm102.1$ for constant (p=0.038), $B=-2.0\pm0.6$ for EDR with $\beta=-0.608$ (p=0.019) and $B=0.08\pm0.03$ for MC with $\beta=0.465$ (p=0.047). Hence, HbA1c did not reach significance as an independent predictor in either model.

Finally we analyzed whether baseline parameters might predict for other indices of renal injury, including FGS, kidney weight and blood pressure (Table 2). A higher body weight at baseline was the only parameter significantly predicting a higher degree of FGS (Table 2) and HbA1c was the only parameter measured significantly predicting a higher future kidney weight (Figure 4B). None of the baseline parameters significantly predicted blood pressure at the end of the study period.
Table 2. Linear regression analysis of baseline parameters and renal outcome parameters.

<table>
<thead>
<tr>
<th>Baseline parameters (assessed at the time of UNX)</th>
<th>Renal outcome parameters after UNx</th>
<th>Proteinuria</th>
<th>FGS (at 10 wks)</th>
<th>SBP (at 10 wks)</th>
<th>KW (at 10 wks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Early increase (0-2 wks)</td>
<td>Gradual increase (2-10 wks)</td>
<td>Overall increase (0-10 wks)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (BW)</td>
<td>-0.197 (ns)</td>
<td>0.462 (ns)</td>
<td>0.303 (ns)</td>
<td><strong>0.822</strong> (ns)</td>
<td>0.092 (ns)</td>
</tr>
<tr>
<td>Renal artery function EDR</td>
<td>-0.490 (ns)</td>
<td>-0.603 (ns)</td>
<td>-<strong>0.856</strong> (ns)</td>
<td>-0.341 (ns)</td>
<td>-0.144 (ns)</td>
</tr>
<tr>
<td>Myogenic constriction (MC*)</td>
<td>0.350 (ns)</td>
<td><strong>0.757</strong> (p=0.030)</td>
<td><strong>0.789</strong> (p=0.020)</td>
<td>-0.187 (ns)</td>
<td><strong>0.537</strong> (p=0.020)</td>
</tr>
<tr>
<td>Hemoglobin A$_1c$ (HbA$_1c$)</td>
<td>-0.053 (ns)</td>
<td><strong>0.886</strong> (p=0.001)</td>
<td><strong>0.780</strong> (p=0.008)</td>
<td>0.263 (ns)</td>
<td>0.234 (ns)</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.209 (ns)</td>
<td>-0.378 (ns)</td>
<td>-0.218 (ns)</td>
<td>-0.240 (ns)</td>
<td>0.034 (ns)</td>
</tr>
<tr>
<td>Plasma creatinine (P$_{Cr}$)</td>
<td>0.341 (ns)</td>
<td>-0.045 (ns)</td>
<td>0.169 (ns)</td>
<td>-0.138 (ns)</td>
<td>-0.041 (ns)</td>
</tr>
<tr>
<td>Creatinine clearance (C$_{Cr}$)</td>
<td>-0.419 (ns)</td>
<td>-0.084 (ns)</td>
<td>-0.379 (ns)</td>
<td>-0.126 (ns)</td>
<td>-0.383 (ns)</td>
</tr>
<tr>
<td>Protein excretion (Prot. Excr.)</td>
<td>0.256 (ns)</td>
<td>-0.374 (ns)</td>
<td>-0.186 (ns)</td>
<td>-0.205 (ns)</td>
<td>0.168 (ns)</td>
</tr>
</tbody>
</table>

Abbreviations: UNx, unilateral nephrectomy; BW, body weight; EDR, endothelium-dependent relaxation; KW, kidney weight; MC, myogenic constriction; P$_{Cr}$, plasma creatinine; C$_{Cr}$, creatinine clearance; Prot. Excr., protein excretion; FGS, focal glomerulosclerosis; SBP, systolic blood pressure; ns, non-significant. BW is g, EDR is AUC in arbitrary units, MC is AUC in arbitrary units, HbA$_1c$ is %, cholesterol is mmol/L, P$_{Cr}$ is μmol/L, C$_{Cr}$ is ml/min/100g, Prot.Excr. is mg/24h; proteinuria is increase in Prot. Excr. from 0-2, 2-10 or 0-10 weeks after UNx,
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FGS is %, SBP is mmHg, KW is mg/g body weight. Data represent r for univariate linear regression analysis with significant p-values in bold as indicated, or best non-significant correlation for a given outcome parameter in bold-italic.

Figure 4. Scatterplots of baseline HbA\(_1c\) with baseline intrarenal artery myogenic constriction (MC) and future kidney weight in young obese ZDF rats. Baseline HbA\(_1c\) positively correlated with intrarenal artery MC at baseline (panel A) and predicted the relative kidney weight following uninephrectomy (UNx) and diabetes mellitus in obese ZDF rats. Fitted lines show the result of linear regression analysis with r- and p-values as indicated.

Discussion

UNx in young obese ZDF at the early onset of DM induced an initial (UNx-driven) steep rise in proteinuria followed by a gradual (DM-driven) increase thereafter which was correlated with the variability in EDR and MC of intrarenal arteries at the start of the study. The present findings demonstrate that baseline intrarenal artery function predicted proteinuria as an indices of renal injury in obese subjects prone to develop DM.

4.1. Augmented proteinuria increase following UNx in young ZDF rats; are obese subjects more susceptible to renal damage development?

In the ZDF rat model, development of DM induces renal injury and progressive proteinuria [17]. Here we additionally performed UNx to assess intrarenal artery function at the start of the study in young obese ZDF rats. Although UNx imposes increased filtration load to the
remnant kidney this normally does not lead to renal injury and development of proteinuria in otherwise healthy individuals due to renal reserve [18,19]. Consistent with that age-matched lean control rats did not develop proteinuria when subjected to UNx. Hence, this renal reserve is also a crucial premise for living kidney donor transplantation. In young obese ZDF rats, however, UNx caused an early steep rise in proteinuria within 2 weeks. This while the normal range metabolic parameters in young ZDF rats indicate that they were (still) at the early onset of DM at the time of UNx. Since it was also lacking in lean-UNx rats the results suggest that the steep rise in proteinuria following UNx was particular for obese subjects.

To our knowledge the effects of UNx on proteinuria in ZDF rats have not been investigated previously. The closest may be a more recent study by Sebeková et al. [20] who assessed proteinuria in Zucker Fatty (ZF) rats at 8 weeks following UNx. Hence, ZF rats over time also develop features such as obesity, hyperglycemia and dyslipidemia, but not typically DM as in ZDF. In that study, proteinuria was ~26 mg/24h in ZF-UNx, compared to ~6 mg/24h in lean-UNx [20], but this is still >7-fold lower than in ZDF-UNx and >4.5-fold lower than in ZDF-sham in the present study. Furthermore, UNx in the study by Sebeková et al. was induced in ZF rats at 4 weeks of age when body weight was (still) similar to that of age-matched lean controls [20]. So one important difference with respect to the present study seems to be the presence of obesity in young ZDF, augmenting the impact of UNx on susceptibility to renal injury as it seems. A second difference is the development of DM in ZDF, but not in ZF rats, accounting for the larger (gradual and overall) increase in proteinuria in ZDF compared to ZF. Interestingly, a higher body weight at baseline was correlated with a higher degree of future FGS (i.e. structural renal damage) in the present study. Taken together, the results suggest that obese subjects at the early onset of DM have augmented susceptibility for renal injury and development of proteinuria. This may generally adhere to the conclusion by Hernandez et al. [21] and others [22] that the metabolic syndrome - which includes obesity - should be considered an contraindication to donation because it is unclear how much risk there is for individuals who donate a kidney and then develop (renal) complications. Hence, according to the analysis of the American registry about 20% of the living donors presents obesity [23]. The tricky point obviously remains that - despite careful prescreening and close follow up care procedures - a uniform prediction or accountability of an individual's future development of obesity and/or other metabolic syndrome features remains uncertain.
4.2. Future development of proteinuria in young obese ZDF rats predicted by baseline intrarenal artery function; consistent predictive value of EDR but not of MC?

To assess whether variation in intrarenal artery function would predict for the subsequent development of renal damage during DM we measured EDR and MC in small arteries obtained from extirpated kidney of young ZDF rats at the early onset of DM. EDR significantly predicted for the overall progression of proteinuria after UNx and development of DM in obese ZDF. Although EDR did not significantly predict for either the early- (i.e. UNx-driven) or the gradual- (i.e. DM-driven) rise in proteinuria, a trend towards a negative correlation between EDR and proteinuria was clearly observed here as well. Nevertheless, the results ultimately suggest that the predictive properties of EDR in young obese ZDF on overall proteinuria increase following UNx and DM development was dependent on the combination of the early rise plus gradual rise in proteinuria. Taken together, the present results further fuel the concept that an intact endothelial dilatory function generally provides a powerful defense mechanism against susceptibility for renal injury and progressive renal damage [6,14] and has a consistent predictive value including in DM.

Next to EDR, intrarenal artery MC also predicted the overall increase of proteinuria in our model with similar correlation coefficients. Since we did not observe a correlation between MC and the early rise in proteinuria nor a trend thereto, we therefore conclude that the predictive properties of MC on the overall rise in proteinuria in our model was mainly mediated through the gradual component of proteinuria development, i.e. during the development of DM. Of importance, MC correlated positively with proteinuria in the present study which is in contrast to our previous findings [11,13]. But in those studies renal damage developed in the presence of HT. Hence, intact (or augmented) MC is believed to protect from an increase in intra-glomerular pressure in rats with elevated blood pressure [10,24]. Indeed, failure to maintain MC is associated with enhanced renal damage development in the hypertensive Fawn-Hooded rat, amongst others [11,12]. *Vice versa*, more pronounced MC of the pre-glomerular arteries is suggested to longer protect the kidneys from HT-damage in spontaneous hypertensive rats (SHR) [24]. However, as obese ZDF do not develop HT – at least not within the study period in the present study - the role of renal artery MC in non-HT models of renal injury seems less clear.

4.3. Other baseline parameters predicting future renal damage in obese ZDF rats developing DM.
Apart from intrarenal artery EDR and MC, baseline HbA₁c was the only additional parameter predicting future proteinuria. Similar to EDR and MC, baseline HbA₁c varied among individuals but was still within the normal range at the start of our study. Interestingly, baseline HbA₁c also positively correlated with baseline MC (Figure 4A). At present, the mechanism behind a possible interaction between MC and HbA₁c is unknown. Previously, it has been shown that augmented oxidative stress and enhanced intrarenal angiotensinogen precede the development of renal injury in the ZDF rat [25]. Additional reports suggest that reactive oxygen species generated from nox2-based NADPH oxidase may augment intrarenal artery MC in SHR, and also AT1-receptor activity has been linked to increased MC reactivity [26,27]. Furthermore, obesity per se is associated with oxidative stress and also glucose levels – as reflected by HbA₁c [28] – play a role in determining oxidative status [29]. The variations in baseline MC in association with baseline HbA₁c in the present study may thus have reflected inter-individual variation in oxidative status at the early onset of DM in young obese ZDF.

Higher basal HbA₁c levels also predicted for increased kidney-to-body weight ratio as an indices of renal injury. Increased kidney weights has been reported previously in ZDF at 16 weeks of age [30]. Clinical studies have demonstrated that the combination of HbA₁c and fasting glucose predict for the development of DM. Results of the present study suggest that the predictive value of basal, normal-range HbA₁c may even be extended to the progression of renal injury after development of DM.

**Conclusions, limitations and perspectives**

Proteinuria increase following UNx was augmented in young obese ZDF rats at the early onset of DM. Although this in itself was a secondary finding it does refer to previous suggestions [31] that obesity in otherwise healthy subjects may increase susceptibility to renal damage. In contrast, blood pressure was not increased following UNx. It should be acknowledged, however, that hemodynamic parameters were obtained under anesthesia which might have limited the detection of potential mild increases in blood pressure to some extent.

Previous proof of principle studies indicated that baseline variation in intrarenal artery function could have a predictive value for an individual's susceptibility to renal injury and CKD [8,11,13,14]. However, the findings of those studies are limited by the fact that renal injury and CKD was induced experimentally rather than the result of natural or clinical
courses such as in DM. As a primary objective, we here found that smaller EDR in young ZDF rats - in which development of DM is accompanied by functional and morphological kidney damage [17] - independently predicted increased future proteinuria. It thereby adheres to the concept that an intact endothelial dilatory function generally provides a powerful defense mechanism against susceptibility for end-organ damage, including renal injury and progressive renal damage in DM. Given the consistency with which intrarenal artery EDR predicted proteinuria, future studies may designed to test whether early modulations aimed at the reinforcement of “normal” endothelial function also attenuate an individual’s susceptibility to renal damage in obese subjects prone to develop DM.

Baseline variation in intrarenal artery MC also predicted future proteinuria in the present study but its direction (i.e. a larger MC predicted for more proteinuria during DM) was opposite from that observed previously. Thus in contrast to EDR, the predictive value of intrarenal artery MC for renal injury seems less consistent which suggests for further investigations hereof. It should additionally be noted here that - given the alleged protective role of intact MC against glomerular damage from barotrauma - such future studies should have a particular notion regarding the absence (i.e. as in the present study) or presence (i.e. as in those previous studies) of HT in DM for clear interpretation and comparisons.
Chapter 2

References


Baseline Intrarenal Artery Function Predicts Proteinuria in Diabetic ZDF Rats