Role of Podocytes for Reversal of Glomerulosclerosis and Proteinuria in the Aging Kidney After Endothelin Inhibition

Jana Ortmann, Kerstin Amann, Ralf P. Brandes, Matthias Kretzler, Klaus Münter, Niranjan Parekh, Tobias Traupe, Melanie Lange, Thomas Lattmann, Matthias Barton

Abstract—The cause of focal-segmental glomerulosclerosis as a consequence of physiological aging, which is believed to be inexorable, is unknown. This study investigated whether inhibition of endothelin-1, a growth-promoting peptide contributing to renal injury in hypertension and diabetes, affects established glomerulosclerosis and proteinuria in the aged kidney. We also determined the role of endothelin receptors for podocyte injury in vivo and in vitro. Aged Wistar rats, a model of spontaneous age-dependent glomerulosclerosis, were treated with the orally active endothelin subtype A (ET\textsubscript{A}) receptor antagonist darusentan, and evaluation of renal histology, renal function studies, and expression analyses were performed. In vitro experiments using puromycin aminonucleoside to induce podocyte injury investigated the role of ET\textsubscript{A} receptor signaling for apoptosis, cytoskeletal injury, and DNA synthesis. In aged Wistar rats, established glomerulosclerosis and proteinuria were reduced by $>50\%$ after 4 weeks of darusentan treatment, whereas blood pressure, glomerular filtration rate, or tubulo-interstitial renal injury remained unaffected. Improvement of structural injury in glomeruli and podocytes was accompanied by a reduction of the expression of matrix metalloproteinase-9 and p21\textsuperscript{Cip1/WAF1}. In vitro experiments blocking ET\textsubscript{A} receptors using specific antagonists or RNA interference prevented apoptosis and structural damage to podocytes induced by puromycin aminonucleoside. In conclusion, these results support the hypothesis that endogenous endothelin contributes to glomerulosclerosis and proteinuria in the aging kidney. The results further suggest that age-dependent glomerulosclerosis is not merely a “degenerative” but a reversible process locally confined to the glomerulus involving recovery of podocytes from previous injury. (Hypertension. 2004; 44:974-981.)

Key Words: arterial pressure ■ nephrosclerosis ■ DNA ■ kidney failure ■ renal artery ■ expression ■ kidney ■ renal disease

Aging represents an important factor determining onset and course of disease and has become a significant issue in view of the anticipated increase of the aging population. Aging in humans and rodents progressively impairs renal function and structure, the latter of which is characterized by damage of podocytes and mesangial matrix, as well as capillary hypertrophy and obliteration resulting in glomerulosclerosis. The exact mechanisms underlying age-dependent renal injury are unknown. In otherwise healthy individuals $\geq 65$ years of age, even in the absence of known risk factors such as hypertension or diabetes, glomerulosclerosis is frequently present. Currently, $\approx 1.4\%$ of the US total population is affected, and the incidence is expected to increase to $>2\%$ within the next 15 years. Glomerulosclerosis and proteinuria involve injury of podocytes, also known as glomerular epithelial cells that maintain an intact filtration barrier and control glomerular basement membrane turnover under normal conditions. In addition to cell-specific changes during aging, cell cycle inhibitors such as p21\textsuperscript{Cip1/WAF1} have been associated with cellular senescence and cell growth and have been linked to glomerular injury.

Endothelin-1 (ET-1), a mitogen and vasoconstrictor signaling via G-protein–coupled endothelin subtype A (ET\textsubscript{A}) and ET\textsubscript{B} receptors, contributes to growth of glomerular mesangial cells. Expression of ET-1 increases in diseased glomeruli and prevention studies indicate that inhibition of ET\textsubscript{A} receptors retards the progression of glomerulosclerosis. As we have shown previously, ET-1 expression increases in the aging kidney in the absence of other risk factors. Because podocytes are targets of ET-1, we sought to investigate the effects of treatment with an orally active ET\textsubscript{A} receptor antagonist on renal...
structure and function in aged rats with established glomerulosclerosis and podocyte injury.

Methods

In Vivo Studies

Animal Experiments
Male Wistar rats (IFFA Credo/Charles River) were obtained at the ages of 1 month and 22 months. At 23 months of age, 8 animals were euthanized and kidneys removed. At 2 and 23 months, animals (9 to 10 per group) were randomized to 28 days of treatment with or without the orally active ETA receptor antagonist darusentan (LU135252; 20 mg/kg per day; Knoll AG) administered in the drinking water. On the day before euthanization, 24-hour metabolic studies were performed as described. In a subset of animals (n=6 per group), glomerular filtration and hemodynamics were determined on the day of euthanization, and the left kidney was perfusion-fixed at mean arterial pressure by glutaraldehyde infusion. Studies were in accordance with the institutional guidelines and approved by the institutional animal care committee.

Blood Pressure, Renal Blood Flow, and Glomerular Filtration Measurements
Measurements of arterial blood pressure and renal blood flow were performed in anesthetized animals using an intra-arterial catheter and a flow probe, respectively. Glomerular filtration rate was determined using the single injection technique as described by Hall et al. For details, see the online supplement, available at http://www.hypertensionaha.org.

Assessment and Quantification of Renal Injury
Renal damage was as described. Glomerular volume was determined by measuring glomerular surface area in sections of pressure-fixed kidney by planimetry. See online supplement for details.

Glomerular Gene Expression In Vivo
Real-time polymerase chain reaction (PCR) was performed from RNA obtained from laser-dissected glomeruli of the study animals. For details, see the online supplement.

Western Blot Analysis and Immunohistochemistry
Western blot analysis of p21<sup>Cip1/WAF1</sup> and p27<sup>kip1</sup> was performed from frozen renal cortices of the study animals. In situ immunohistochemistry of p21<sup>Cip1/WAF1</sup> was performed on renal cryosections. See the online supplement for details.

In Vitro Studies

Podocyte Injury
Puromycin aminonucleoside–induced podocyte injury was used to investigate the effects of drug treatment and gene silencing. For cytoskeleton organization experiments, mouse podocytes were used. See the online supplement for details.

Gene Expression Analysis
Expression of mRNA in human podocytes was determined as described previously using quantitative real-time PCR. See the online supplement for details.

Gene Silencing
RNA interference experiments in human podocytes were performed as described by Tuschl after transfection of small interfering RNA (siRNA) or nonsilencing RNA using Lipofectamine 2000 (Invitrogen). See the online supplement for details.

DNA Synthesis
In quiescent cultured human podocytes, DNA synthesis was studied as described previously. See the online supplement for details.

Statistical Analyses
Data are means [SD], and n denotes the number of animals or independent in vitro experiments, respectively. Data were analyzed using ANOVA with Bonferroni correction or the Mann–Whitney U test when appropriate. A P value <0.05 was considered significant.

Results

Focal-segmental and global glomerulosclerosis including podocyte injury, mesangial matrix expansion, capillary occlusion, and tuft adhesion (Figure 1a) were present in aged rats and comparable in animals aged 23 and 24 months (score 0.83 [0.17] and 0.87 [0.11], respectively; P=0.56). Hyper-
Figure 2. Representative transmission electron micrographs of podocytes and glomerular basement membranes from untreated (top panels) and darusentan-treated aged kidneys (bottom panels). a, Untreated, showing glomerular basement membrane hypertrophy and podocyte injury and detachment. b, Untreated, high-power micrograph demonstrating thickening of glomerular capillary basement membrane with podocyte detachment. Injury of podocytes is characterized by hypertrophy, inclusion of cytoplasmatic absorption droplets, and diffuse effacement of foot processes. c, Darusentan treatment showing attachment of the podocyte to the basement membrane and reversal of glomerular capillary hypertrophy. d, High-power micrograph showing podocyte recovery after darusentan treatment. Endothelin blockade is associated with a reduction of podocyte injury and regression of absorption droplets. P indicates podocyte.

Effects of Darusentan Treatment for 28 Days in Young and Aged Rats on Physiology and Renal Parameters

<table>
<thead>
<tr>
<th>Group Treatment</th>
<th>Young Untreated</th>
<th>Young Darusentan</th>
<th>Old Untreated</th>
<th>Old Darusentan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distribution volume</td>
<td>0.29 [0.02]</td>
<td>0.30 [0.03]</td>
<td>0.20 [0.02]*</td>
<td>0.21 [0.02]*</td>
</tr>
<tr>
<td>Plasma renin activity (µg/L per hour)</td>
<td>22.6 [9.8]</td>
<td>23.3[8.5]</td>
<td>5.3 [4.4]</td>
<td>7.3 [5.9]</td>
</tr>
<tr>
<td>2K-GFR (mL/minute)</td>
<td>4.3 [0.2]</td>
<td>4.8 [0.6]</td>
<td>5.4 [0.5]*</td>
<td>5.6 [0.6]*</td>
</tr>
<tr>
<td>RBF (mL/minute)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>7.64 [1.42]</td>
<td>7.0 [1.21]</td>
</tr>
</tbody>
</table>

Distribution volume is expressed in absolute values for exogenous creatinine (mL) as fraction of body volume (grams of body weight in mL). Data are means [SD].

n.d. indicates not done; 2K-GFR, 2K-glomerular filtration rate; RBF, renal blood flow.

*P<0.01 vs young.
receptor antagonist darusentan for 28 days. Treatment markedly reduced established glomerulosclerosis (from 0.876 [0.09] to 0.4 [0.09]; 55% inhibition; \( P \leq 0.0008 \); Figures 1b and 3a) and proteinuria (from 307 [153] to 130 [89] mg/kg per day; 57% inhibition; \( P = 0.0101 \); Figure 3b), and reversed podocyte injury and glomerular basement membrane hypertrophy (Figure 2c and 2d). In aged rats, treatment had no effect on tubulo-interstitial injury (injury score 1.18 [0.31] versus 1.26 [0.34]; \( P = 0.7 \); Figure 1d), glomerular size (untreated 13 039 [783]; darusentan 12 011 [1895] \( \mu \text{m}^2 \); \( P = 0.178 \)), plasma renin activity, mean arterial blood pressure, and glomerular filtration rate (NS; Table). Darusentan treatment also had no effect on plasma or urinary creatinine levels (NS; Table).

To determine the effect of treatment on gene and protein expression, we next analyzed expression of the cyclin-dependent kinase inhibitor p21CIP1/WAF1, which also contributes to cell differentiation, senescence, and glomerulosclerosis.\(^9,10\) Protein expression of p21CIP1/WAF1 was hardly detectable by Western blot analysis (Figure 3c and 3e) or immunostaining in glomeruli and podocytes of young rats (Figure 4a), whereas expression increased strongly in aged rats (8.5-fold, from 1.8 [1.2] to 14.8 [4.0] optical density [OD] units; \( P = 0.001 \); Figure 3c and 3e), being particularly localized to glomeruli and podocytes (Figure 4b). After darusentan treatment, expression was markedly reduced (from 14.8 [2.0] to 5.0 [1.2] OD units; 65% inhibition; \( P = 0.006 \); Figures 3c and 3e and 4c), and glomerular and podocyte expression of p21CIP1/WAF1 was similar to that in young animals (Figure 4c). Increase of the cyclin-dependent kinase inhibitor p27KIP1 expression in aged kidneys was unaffected by darusentan treatment (Figure 4a).
3d). We also determined the effects of darusentan treatment for glomerular gene expression in vivo of matrix metalloproteinase-9 (MMP-9), which is involved in glomerular matrix turnover.5 MMP-9 gene expression was determined in laser-dissected glomeruli of the study animals and was found to be reduced by 65% after darusentan treatment (from 6.0 [2.8] to 2.1[1.6] units; n/11005 10 per group; P/11005 0.0015; Figure 5a) because stability and function of podocytes were improved after darusentan treatment in vivo and regulation of calcium signaling by endothelin15 suggests that podocytes are functional targets of ET-1. We set out experiments to investigate the role of endogenous and exogenous endothelin in an in vitro podocyte injury model. Endothelin receptor expression of both endothelin receptor subtypes was present in human podocytes (Ortmann and Barton, unpublished observation, 2004), and MMP-9 gene expression and cytoskeletal disruption were chosen as read-outs of podocyte injury as described previously.5,23,27 As expected, puromycin injury increased MMP-9 gene expression (507%; n=6 per group; P=0.0041; Figure 5c) and caused podocyte shrinkage and cytoskeleton disruption (Figures 5f and 6c and 6d).27 MMP-9 gene induction was completely prevented by pretreatment with different ETA receptor antagonists BQ-123 (peptide; 10 nmol/L) or darusentan (LU135252 [LU]; 30 nmol/L) on podocyte MMP-9 gene expression after puromycin injury. Cells in control and puromycin only experiments (20 μg/mL) had been transfected with nonsilencing RNA duplex (ncRNA). e, Effect of ETA receptor siRNA or control experiments using nonsilencing RNA siRNA on de novo DNA synthesis in quiescent human podocytes. f, Effects of ETA receptor antagonists BQ-123 (peptide; 10 nmol/L) or LU135252 (darusentan, nonpeptide; 30 nmol/L) on podocyte cytoskeleton disruption after puromycin injury (10 μg/mL) quantified from pooled mouse podocyte experiments as shown in Figure 6. All PCRs were run in triplicate. O indicates old; OLU, old, darusentan; PAN, puromycin aminonucleoside. *P<0.05 vs CTL; †P<0.05 vs PAN alone/old.

Figure 5. a, Gene expression of MMP-9 from laser-dissected glomeruli of aged rats without and after darusentan treatment. Gene expression levels are given as arbitrary units (AU) using the ΔΔCT method. b, Effect of ETA receptor blockade on podocyte function in vitro. Puromycin aminonucleoside (50 μg/mL)-induced apoptosis in human podocytes (P<0.05 vs control [CTL]). c, Effect of selective ETA receptor antagonists BQ-123 (BQ; 10 nmol/L) or darusentan (LU135252 [LU]; 30 nmol/L) on podocyte MMP-9 gene expression after puromycin injury. d, Effect of ETA receptor RNA interference (siRNA) on podocyte MMP-9 gene expression after puromycin injury; cells in control and puromycin only experiments (20 μg/mL) had been transfected with nonsilencing RNA duplex (ncRNA). e, Effect of ETA receptor siRNA or control experiments using nonsilencing RNA (ncRNA) on de novo DNA synthesis in quiescent human podocytes. f, Effects of ETA receptor antagonists BQ-123 (peptide; 10 nmol/L) or LU135252 (darusentan, nonpeptide; 30 nmol/L) on podocyte cytoskeleton disruption after puromycin injury (10 μg/mL) quantified from pooled mouse podocyte experiments as shown in Figure 6. All PCRs were run in triplicate. O indicates old; OLU, old, darusentan; PAN, puromycin aminonucleoside. *P<0.05 vs CTL; †P<0.05 vs PAN alone/old.
**Discussion**

Renal aging is associated with renal disease and nonrenal clinical complications in humans. Using a suitable animal model, we report partial restoration of glomerular structure in association with a reversal of proteinuria and glomerular expression of genes indicative of injury and senescence after ETA receptor blockade. Protection from injury was also observed after inhibition of ETA receptor signaling in an in vitro model of podocyte injury using puromycin aminonucleoside. Our results suggest that endothelin contributes to spontaneous glomerular injury associated with renal aging. This was found to be a reversible process involving changes in podocyte structure mediated through ETA receptor activation.

The findings reported here were obtained in normotensive animals and independent of blood pressure, glomerular filtration rate, renal blood flow, or tubulo-interstitial changes. Consistent with these results, preliminary data from Rabelink’s group indicate that endothelin blockade reduces proteinuria in diabetic patients with a normal glomerular filtration rate. Similar to our study, these investigators found the reversal of established proteinuria to be unrelated to blood pressure, glomerular filtration rate, or renal blood flow. The exact mechanisms leading to reversal of established proteinuria after ETA receptor blockade are presently unclear. However, the results presented here strongly support a role for endogenous endothelin and podocyte function, which may be similarly relevant for the development of proteinuria in diabetics. Further studies are needed to clarify the exact mechanism(s) by which different drug treatments can reverse glomerular disease.

We have demonstrated previously that aging activates the renal endothelin system. This activation may be, in part, dependent on angiotensin II. Indeed, early studies by Remuzzi’s group have shown that inhibition of angiotensin II formation with the angiotensin-converting enzyme (ACE) inhibitor perindopril for 4 months slowed development of glomerular and tubulo-interstitial injury in aging rats, effects that were accompanied by a pressure-lowering effect of the drug. In contrast to these studies, blockade of ET receptors in our experiments had no effect on blood pressure or plasma renin activity, but substantially reversed established glomerulosclerosis and proteinuria without modifying tubulo-interstitial injury within 4 weeks of treatment. These new findings suggest that (1) podocytes can undergo substantial structural recovery after injury has occurred, and (2) podocyte recovery results in selective restoration of kidney structure and function that is locally confined to the glomerulus. Given that previous regression studies observed improvement in tubulo-interstitial injury only after pressure-lowering drug treatment, and that in our study, tubulo-interstitial injury and glomerular size were unaffected by treatment, we propose local, pressure-independent mechanisms to be involved in the reversal of glomerulosclerosis, as proposed for the antiproteinuric effects of ACE inhibition. Moreover, we observed recently that in mice with focal-segmental glomerulosclerosis, improvement of tubulo-interstitial injury by drug treatment may occur while glomerular injury and proteinuria persist. This further suggests independence of disease processes in the glomerulus from those occurring in the tubulo-interstitium.

Although unable to complete cell division, podocytes may re-enter cell cycle and undergo mitosis or nuclear division under certain conditions. This ability of podocytes to re-enter the cell cycle would also be supported by the observation that...
in cultured podocytes, DNA synthesis increased after inhibition of ETα receptors, indicating an inhibitory effect of endogenous endothelin on podocyte cell cycle activity. In the kidney, expression of the cyclin-dependent kinase inhibitor p21Cyclin/ WAF1 increases with injury and aging.9,10,35,36 Moreover, p21Cyclin/ WAF1 directly contributes to renal injury because its deletion conveys protection from glomerulosclerosis.10 Indeed, in addition to its role as a cell cycle inhibitor, p21Cyclin/ WAF1 is involved in cellular senescence and differentiation.8,37 Induction of p21Cyclin/ WAF1 expression in podocytes occurs in murine and human glomerulosclerosis10,35 and, as shown in the present study, in aged glomeruli. Because we observed a strong reduction of p21Cyclin/ WAF1 expression after darusentan treatment, structural recovery of renal injury after darusentan treatment may be, at least in part, related to effects of endothelin blockade on cell cycle or cell differentiation during the aging process, which appear to be regulated in a reversible manner.

In the present study, we analyzed gene expression of MMP-9 in glomeruli of the study animals as well as in cultured podocytes, which was found to be downregulated after ETα receptor blockade in vitro and in vivo. MMP-9, also known as gelatinase B, degrades collagen38 and thus contributes to glomerular matrix turnover.5,39 We found normalization of hypertrophy of the glomerular basement membrane in aged rats as detected by electron microscopy after endothelin blockade. Although we did not measure the activity of MMP-9 or other MMPs, or their respective inhibitors,30 these blockades may have occurred because of a reduced degradation of mesangial collagen and reduction of glomerulosclerosis recently described for other drugs.29,30 Finally, our observation that podocyte injury was induced by exogenous ET-1 and that injury by the unspecific stimulus pyruvycin was prevented by ETα receptor blockade indicates that intracellular and extracellular ET-1 contribute to injury. This notion is supported by preliminary data by Morigi et al, who observed induction of ET-1 gene expression and cytoskeleton disruption in mouse podocytes in response to indirect injury after exposure to protein overload in vitro.40

In summary, the results support the hypothesis that endogenous endothelin contributes to glomerulosclerosis and proteinuria in the aging kidney. Our results further suggest that age-dependent glomerulosclerosis is not merely a degenerative but a locally confined reversible process enabling podocytes to recover from previous injury. If operative in humans, reversal of glomerular disease by endothelin inhibition could improve treatment of established renal diseases and their clinical complications in patients.

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References


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