Endothelin-1 Induces Proteinuria by Heparanase-Mediated Disruption of the Glomerular Glycocalyx

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ABSTRACT

Diabetic nephropathy (DN) is the leading cause of CKD in the Western world. Endothelin receptor antagonists have emerged as a novel treatment for DN, but the mechanisms underlying the protective effect remain unknown. We previously showed that both heparanase and endothelin-1 are essential for the development of DN. Here, we further investigated the role of these proteins in DN, and demonstrated that endothelin-1 activates podocytes to release heparanase. Furthermore, conditioned podocyte culture medium increased glomerular transendothelial albumin passage in a heparanase-dependent manner. In mice, podocyte-specific knockout of the endothelin receptor prevented the diabetes-induced increase in glomerular heparanase expression, consequent reduction in heparan sulfate expression and endothelial glycocalyx thickness, and development of proteinuria observed in wild-type counterparts. Our data suggest that in diabetes, endothelin-1 signaling, as occurs in endothelial activation, induces heparanase expression in the podocyte, damage to the glycocalyx, proteinuria, and renal failure. Thus, prevention of these effects may constitute the mechanism of action of endothelin receptor blockers in DN.


Diabetic nephropathy (DN), a major complication of both type 1 and type 2 diabetes mellitus, is the leading cause of CKD in the Western world. DN is characterized by a progressive increase in proteinuria, glomerular capillary widening, secondary podocyte loss, mesangial matrix expansion, glomerular basement membrane thickening, and finally, tubulointerstitial fibrosis. Current treatment does not reduce proteinuria sufficiently, therefore, there is a need to identify new therapeutic targets.

One potent new therapeutic target is the endothelin-1 receptor. Endothelin-1 is a vasoconstrictor that is released upon endothelial activation and activates two G-protein-coupled receptors, endothelin receptor type A (ETRA) and endothelin receptor type B (ETRB). Together, these receptors induce a variety of intracellular signaling cascades, resulting in vasoconstriction, proliferation, inflammation, extracellular matrix production, and fibrosis.1–4 The expression of endothelin-1 is increased in DN.5 Several studies report a renoprotective effect of endothelin receptor antagonists (ERAs) in both experimental as well as clinical DN.6–8 Moreover, ERAs reduce residual proteinuria and improve endothelial function in patients with DN, in addition to the standard treatment.5,9 ERAs also reduce proteinuria in other forms of nephropathy, such as hypertensive nephropathy and FSGS.10 It was shown that podocyte-specific deletion of both ETRA and ETBR protects mice from diabetes-induced glomerulosclerosis and podocyte loss, indicating that endothelin-1 signaling in the podocyte may play a role in the development of DN and the protective effects of endothelin blockers.11 The mechanism underlying the protective effect of ERAs, however, remains unknown.

We previously showed that heparanase is essential for the development of DN.12 Heparanase-deficient mice were protected from diabetes-induced proteinuria and renal damage. Heparanase is the only known mammalian enzyme that can cleave the negatively charged

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heparan sulfate (HS) side chains of HS proteoglycans. Loss of HS expression in the glomerular filtration barrier has been associated with the development of proteinuria. In addition, HS is the major functional glycosaminoglycan in the endothelial glycocalyx, and remodeling of the glycocalyx is associated with the development of proteinuria.

Based on our previous data, we hypothesize that endothelin-1 signaling modulates the endothelial glycocalyx in DN through heparanase release by the podocyte. In this study, we evaluate the effects of endothelin-1 on heparanase expression and function both in vitro and in vivo.

First, we evaluated the effects of endothelin-1 on cultured podocytes and glomerular endothelial cells. Endothelin-1 treatment increased heparanase mRNA expression (Figure 1A) and heparanase activity (Figure 1B) in mouse podocytes and culture supernatant of mouse podocytes (Figure 1C), whereas heparanase expression in cultured mouse glomerular endothelial cells (mGEnC-1) was not affected (Supplemental Figure 1A). Notably, the endothelin-1–induced increase in heparanase activity was larger in the culture supernatant versus the cell extract, respectively, about 100% versus 20%, suggesting that the majority of endothelin-1–induced heparanase is secreted by the podocytes. Treatment of mGEnC-1 with culture supernatant of endothelin-1–stimulated podocytes increased transendothelial albumin passage (Figure 1D and E). Heparanase expression (Figure 1A), heparanase activity (Figure 1B, C, and E), and transendothelial albumin passage (Figure 1D and E) were reduced by treatment with the combined ERA PD 142893 and the ETRA antagonist BQ-123. Addition of the anti-heparanase antibody HPA1 to the conditioned podocyte culture medium also reduced transendothelial albumin passage (Figure 1F), whereas the isotype control had no effect (Figure 1G). As a control, we showed that addition of the anti-heparanase antibody to recombinant heparanase reduced recombinant heparanase activity (Figure 1H), which suggests that the increased transendothelial albumin passage is mediated by heparanase. Moreover, stimulation of mGEnC-1 with bacterial heparinase or recombinant heparanase to remove HS resulted in an increased transendothelial albumin passage (Supplemental Figure 2A and B), suggesting that HS in the glycocalyx contributes to barrier function in the in vitro transendothelial albumin passage system. Stimulation of mGEnC-1 with endothelin-1 without conditioned podocyte culture medium did not affect transendothelial albumin passage (Supplemental Figure 1B). The observed differences between mGEnC-1 and podocytes in response to endothelin-1 can be explained by differences in endothelin receptor expression. Podocytes express both ETRA and ETRB, while glomerular endothelial cells only express ETRB.

To translate our in vitro data, we used podocyte-specific ETRA/ETRB-deficient (podETRKO) mice, in which we induced diabetes. As we reported previously, diabetic podETRKO mice are, in contrast to diabetic wild-type (WT) mice, protected from proteinuria, glomerular basement membrane thickening, mesangial matrix expansion, podocyte loss, and glomerulosclerosis during streptozotocin-induced diabetes. Ten weeks after induction of diabetes, WT mice developed significant albuminuria (Figure 2A), which was significantly reduced in the diabetic podETRKO mice. Diabetic WT mice showed increased cortical heparanase mRNA (Figure 2B), glomerular heparanase protein expression (Figure 2C), and glomerular heparanase activity (Figure 2D), whereas glomerular HS expression was reduced (Figure 2E). In contrast, heparanase and HS expression were normal in the diabetic podETRKO mice (Figure 2, B–E). Next, we analyzed glycocalyx thickness, since increased heparanase expression may reduce glycocalyx thickness in diabetic mice. The glycocalyx was visualized by lanthanum hydroxide staining, which stains the glycocalyx on top of the endothelium and podocytes black. Indeed, glycocalyx thickness on both the endothelium and podocytes was reduced by about 50%–60% in diabetic WT mice (Figure 3, A–C). Importantly, endothelial glycocalyx thickness was preserved in the diabetic podETRKO mice (Figure 3, A and B). Podocyte glycocalyx in diabetic podETRKO mice was reduced by about 25% (Figure 3, A and C), which possibly could be explained by a reduction in HS synthesis, as glomerular heparanase activity is not increased in the diabetic podETRKO mice. Apparently, the remaining reduction of podocyte glycocalyx thickness in podETRKO mice is not sufficient to induce proteinuria and DN. However, we cannot exclude the possibility that a combined reduction of endothelial and podocyte glycocalyx is required for development of proteinuria and DN in WT mice.

Our study is the first to show that endothelin-1 regulates heparanase expression in podocytes. We previously showed that heparanase is essential for the development of proteinuria and renal damage in experimental DN. By induction of diabetes, heparanase-deficient mice showed no mesangial matrix expansion, macrophage infiltration, and tubulointerstitial fibrosis, effects that are known to be mediated by signaling through the endothelin receptor. In addition, we previously showed that endothelin receptor deficiency in podocytes protects mice against experimental DN. In the current study, we show that endothelin-1 induced heparanase expression in mouse podocytes both in vitro and in vivo. Moreover, podocyte-specific loss of both ETRA and ETRB prevented damage to the endothelial glycocalyx after induction of type 1 diabetes.

Our data suggest that crosstalk between podocytes and the glomerular endothelium may be involved in the development of albuminuria and DN. Most extensively studied is the crosstalk of vascular endothelial growth factor and angiopoietin 1 and 2 between podocytes and glomerular endothelial cells, where the glomerular endothelial cell integrity depends upon signaling from the podocyte. A recent study also reported the crosstalk of endothelin-1 between podocytes and glomerular endothelial cells. We recently showed that loss of podocyte heparanase prevents the
Endothelin-1–induced heparanase expression in the podocyte increases transendothelial albumin passage. (A) Heparanase mRNA expression and (B, C) heparanase activity in (A, B) mouse podocytes and (C) culture supernatant of mouse podocytes were increased by treatment with endothelin-1 for 18 hours, and reduced by treatment with the ETRA antagonist BQ-123 and the combined ERA PD 142893. (D, E) Cultured mGEnC-1 were treated with culture supernatant of podocytes treated with endothelin-1 in the absence or presence of (D) BQ-123 or (E) PD 142893. Cumulative passage of FITC-labeled albumin across the mGEnC-1 monolayer (TEER: 28.0 cm⁻²) was determined over time. Transendothelial albumin passage was increased by endothelin-1, and reduced by BQ-123 and PD 142893. To evaluate the role of heparanase on endothelin-1–induced transendothelial albumin passage, (F) the anti-heparanase antibody HPA1 (1:100 and 1:250) and (G) the isotype control rabbit IgG (1:100 and 1:250) were added to the culture supernatant of endothelin-1–treated podocytes. Transendothelial albumin passage was reduced by addition of the anti-heparanase antibody, but not affected by addition of the isotype control. (H) To show that the anti-heparanase antibody HPA1 was able to reduce heparanase activity specifically, recombinant heparanase was coincubated with the antibody (1:100 and 1:250). Heparanase activity was reduced by the anti-heparanase antibody at both tested concentrations. The isotype control had no effect on heparanase activity (not shown). (A, B, C, H) Each bar represents n=4; mean ± SEM; analyzed by a one-way ANOVA. (D, E, F, G) Each dot represents n=3; mean ± SEM; analyzed by a two-way repeated measures ANOVA. *P<0.05, **P<0.01, and ***P<0.001 versus control; #P<0.05, ##P<0.01, and ###P<0.001 versus ET-1. ET-1, endothelin-1; HPSE, heparanase; TEER, transendothelial electrical resistance.
increased passage of albumin across the endothelial monolayer induced by adriamycin. In our current study, we show that heparanase in culture supernatant of endothelin-1–stimulated podocytes is responsible for increased transendothelial albumin passage in vitro, while endothelin-1 had no direct effect on endothelial cells, suggesting podocyte endothelial crosstalk. Notably, our transendothelial albumin flux model does not intend to mimic albumin flux in vivo, but rather is an assay of documenting the effects of endothelin-1 and heparanase on glomerular endothelial cells.

In addition to a potential podocyte-endothelial cell crosstalk, there may be various other mechanisms involved in the reduced endothelial glycocalyx thickness in WT mice. We have previously shown that high glucose, angiotensin II, aldosterone, reactive oxygen species, vitamin D, and inflammatory cytokines such as TNF-α and IL-1β regulate heparanase expression. It could be that in WT mice one or more of these mediators induces endothelial heparanase expression, whereas these mediators are not operative in podETRKO mice or are counteracting each other’s activities.

**Figure 2.** Glomerular heparanase and HS expression are normal in diabetic podETRKO mice. Ten weeks after induction of diabetes by streptozotocin, (A) WT mice developed a significant albuminuria, as indicated by an increased albumin-to-creatinine ratio. Albuminuria was significantly reduced in the diabetic podETRKO mice. (B) Cortical heparanase mRNA expression, (C) glomerular heparanase protein expression, and (D) glomerular heparanase activity were increased by induction of diabetes in WT mice, whereas (E) glomerular HS expression was reduced. (B) Heparanase mRNA expression was significantly lower in the diabetic podETRKO mice, whereas (C) heparanase protein expression, (D) heparanase activity, and (E) HS expression were normal in the diabetic podETRKO mice. n=6–7 mice per group; analyzed using a one-way ANOVA. (C, E) Original magnification, ×400. *P<0.05, **P<0.01, and ***P<0.001 versus nondiabetic control; #P<0.05 and ##P<0.01 versus diabetic WT. AU, arbitrary units.
Another possibility could be that macrophage influx is altered in podETRKO. Macrophages provide cathepsin L, a protease that is required to activate endothelial-derived inactive proheparanase. Finally, podocyte-derived cathepsin L may be reduced in podETRKO mice, thereby limiting activation of endothelial-derived proheparanase. The exact molecular mechanisms underlying the observed differences in endothelial glyocalyx thickness in diabetic WT mice and podETRKO remain unclear.

The glomerular endothelial glyocalyx plays an important role in vascular permeability, attenuation of blood cell–vessel wall interactions, mechanotransduction, signaling, and vascular protection. The podocyte glyocalyx is essential for maintaining foot process and slit diaphragm structure, and is also suggested to play a role keeping a certain distance between podocytes and parietal epithelial cells, thereby helping to maintain glomerular structure and function. Previous studies have reported variations in endothelial glyocalyx thickness, which may be explained by differences in perfusion-fixed staining versus immersion-fixed staining. Very few studies showed podocyte glyocalyx staining and, to the best of our knowledge, we are the first to report the simultaneous quantification of podocyte glyocalyx thickness and glomerular endothelial glyocalyx thickness.

Previous studies showed that a reduced endothelial glyocalyx thickness is associated with the development of proteinuria. Moreover, diabetic Zucker fatty rats showed a reduced endothelial glyocalyx thickness and a significant proteinuria, which was, at least in part, mediated by an increased glomerular heparanase expression. Here, we showed that endothelin-1 signaling in the podocyte increased heparanase expression, thereby most likely reducing endothelial glyocalyx thickness and inducing proteinuria.

In conclusion, endothelin-1 induces heparanase in the podocyte, which may lead to disruption of the glyocalyx and proteinuria in experimental DN, thereby providing a possible mechanistic basis for the renoprotective effects of ERAs. In addition, targeting of heparanase in DN may serve as an alternative.

Figure 3. Glomerular endothelial glyocalyx is preserved in diabetic podETRKO mice. (A) Representative transmission electron microscopy images of lanthanum hydroxide visualizing the glyocalyx on top of the endothelium and podocytes (in black). Scale bar, 500 nm. (B) Quantification of endothelial glyocalyx thickness and (C) quantification of podocyte glyocalyx thickness. Glyocalyx thickness on both the endothelium and podocytes was approximately 50%–60% reduced by induction of diabetes in WT mice. Endothelial glyocalyx thickness was preserved in diabetic podETRKO mice, but podocyte glyocalyx was approximately 25% reduced in diabetic podETRKO mice. Results were analyzed by a one-way ANOVA. ***P<0.001 versus nondiabetic control; ###P<0.001 versus diabetic WT.
therapeutic option for treatment of DN.

CONCISE METHODS

Animals
Mice with podocyte-specific deletion of both Ednar and Ednbr genes (podocin-Cre\(^{−}\) Ednar\(^{lox/lox}\) Ednbr\(^{lox/lox}\) mice) were generated as described.\(^1\) Mice were on a mixed C57BL/6J/129/SV/FVB/N genetic background. Mice homozygous for floxed Ednar and Ednbr genes but without Cre were used as control mice.

To induce diabetes, 12-week old male mice were injected intraperitoneally with 100 mg/kg streptozotocin in a citrate buffer (pH 4.5) for 2 consecutive days, as described.\(^1\) Control mice were injected with citrate buffer alone. Mice with a fasted blood glucose >16 mmol/L were considered diabetic. Mice were euthanized 10 weeks after the induction of diabetes. Urinary creatinine concentrations were measured spectrophotometrically using colorimetric methods. Urinary albumin excretion was measured by ELISA (BIOTREND Chemikalien GmbH, Köln, Germany). Glomeruli were isolated from freshly harvested kidneys as described previously.\(^1\) All animal experiments were performed according to French veterinary guidelines and those formulated by the European Commission for experimental animal use (L358–86/609EEC), and were approved by the Institute National de la Santé et de la Recherche Médicale.

Immunofluorescence Staining
Indirect immunofluorescence staining was performed on 2-μM thick cryosections as described.\(^2\) Primary antibodies included heparanase (HPA1; ProsPecTany, Rehovot, Israel) and the Vesicular Stomatitis Virus- heparanase (HPA1; ProsPecTany, Rehovot, Israel) and the Vesicular Stomatitis Virus–tagged anti-HS antibody HS4C3 (N-, 2-, 3-O, and 6-O sulfation).\(^3\) Secondary antibodies included goat anti-rabbit IgG Alexa 488 (Invitrogen, Carlsbad, CA) and rabbit anti-Vesicular Stomatitis Virus Cy3 (Sigma-Aldrich, St. Louis, MO). At least 50 glomeruli per section were scored for heparanase and HS staining intensities on a scale between 0 and 10 (0 = no staining, 5 = 50% staining, 10 = 100% staining) by two independent investigators on blinded sections using a Leica CTR6000 microscope (Leica Microsystems, Buffalo Grove, IL).

Glycocalyx Staining
Small pieces of cortex were fixed in periodate-lysine-paraformaldehyde (pH 6.2), immersed in 2.3 M sucrose and snap frozen in liquid nitrogen. Frozen sections (20 μm) were stained and fixed with 1% lanthanum hydroxide and 2% glutaraldehyde dissolved in 0.1 M sodium cacodylate buffer (pH 7.4). Samples were washed in 0.1 M sodium cacodylate buffer (pH 7.4), postfixed in Palade-buffered 1% osmium tetroxide with 1% lanthanum hydroxide, washed in 0.1 M sodium cacodylate buffer (pH 7.4), dehydrated, and embedded in Epon 812, Luft procedure (EMS, Hatfield, United Kingdom). Endothelial and podocyte glycocalyx thickness (in nm) was measured in three nondiabetic and four diabetic mice per group. For each mouse, at least ten randomly selected capillary loops of at least five randomly selected glomeruli were analyzed at randomly selected measuring points. The measurements were performed twice by two independent investigators on blinded sections using a Jeol JEM 1400 electron microscope (Jeol, Tokyo, Japan) and Gata microscopy suite.

Cell Culture
Conditionally immortalized mGEnC-1 and mouse podocytes (MPC-5) were cultured as described previously.\(^4\) Differentiated mGEnC-1 and podocytes were treated with endothelin-1 (100 nM; Sigma-Aldrich) and/or the combined ERA PD 142893 (0.1 μM; Sigma-Aldrich) or the selective ETRA antagonist BQ-123 (1 μM; Sigma-Aldrich) for 18 hours.

RNA Isolation and Real-Time PCR
Total RNA was extracted from kidney cortex, mGEnC-1 and MPC-5 podocytes. RNA isolation, cDNA synthesis, and real-time PCR were performed as described.\(^5\)

Heparanase Activity Assay
Heparanase activity of recombinant heparanase, isolated glomeruli, MPC-5 podocytes, and culture supernatant was determined using a commercially available assay (AMS Biotechnology, Abingdon, UK) according to the manufacturer’s instructions.

Transendothelial Albumin Passage and Transendothelial Electrical Resistance Measurement
Differentiated mGEnC-1 seeded on polyester membranes in tissue culture inserts (0.4 μm pore size; Corning, Corning, NY). Transendothelial electrical resistance of untreated cells was measured using a Millicell-ERS2 Volt-Ohm meter (EMD Millipore, Billerica, MA). mGEnC-1 were treated with bacterial heparinase and recombinant heparanase for 1 hour, or with endothelin-1 and/or the combined ERA PD 142893 or the selective ETRA antagonist BQ-123 as outlined. In addition, mGEnC-1 were exposed to culture supernatant of vehicle- or endothelin-1–treated podocytes, and where indicated, substituted with the anti-heparanase antibody HPA1 (ProsPecTany) or the isotype control rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Transendothelial albumin passage was determined as described.\(^6\)

Statistical Analyses
Values are expressed as mean ± SEM. Significance was evaluated by a one-way ANOVA and post hoc analysis with Tukey multiple comparison test. Significant differences in transendothelial albumin passage were evaluated using a two-way repeated measures ANOVA with Bonferroni post-test. Statistical analysis was performed using GraphPad Prism 5.03 (GraphPad Software Inc., San Diego, CA). A P value of ≤0.05 was considered statistically significant.

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DISCLOSURES
None.

REFERENCES


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