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Direct Action of Endothelin-1 on Podocytes Promotes Diabetic Glomerulosclerosis

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ABSTRACT

The endothelin system has emerged as a novel target for the treatment of diabetic nephropathy. Endothelin-1 promotes mesangial cell proliferation and sclerosis. However, no direct pathogenic effect of endothelin-1 on podocytes has been shown *in vivo* and endothelin-1 signaling in podocytes has not been investigated. This study investigated endothelin effects in podocytes during experimental diabetic nephropathy. Stimulation of primary mouse podocytes with endothelin-1 elicited rapid calcium transients mediated by endothelin type A receptors (ETARs) and endothelin type B receptors (ETBRs). We then generated mice with a podocyte-specific double deletion of ETAR and ETBR (NPHS2-Cre \times Ednra^{lox/lox} \times Ednrb^{lox/lox} [Pod-ETRKO]). *In vitro*, treatment with endothelin-1 increased total β -catenin and phospho-NF- κ B expression in wild-type glomeruli, but this effect was attenuated in Pod-ETRKO glomeruli. After streptozotocin injection to induce diabetes, wild-type mice developed mild diabetic nephropathy with microalbuminuria, mesangial matrix expansion, glomerular basement membrane thickening, and podocyte loss, whereas Pod-ETRKO mice presented less albuminuria and were completely protected from glomerulosclerosis and podocyte loss, even when uninephrectomized. Moreover, glomeruli from normal and diabetic Pod-ETRKO mice expressed substantially less total β -catenin and phospho-NF- κ B compared with glomeruli from counterpart wild-type mice. This evidence suggests that endothelin-1 drives development of glomerulosclerosis and podocyte loss through direct activation of endothelin receptors and NF- κ B and β -catenin pathways in podocytes. Notably, both the expression and function of the ETBR subtype were found to be important. Furthermore, these results indicate that activation of the endothelin-1 pathways selectively in podocytes mediates pathophysiologic crosstalk that influences mesangial architecture and sclerosis.

Diabetic nephropathy (DN) is the major microvascular complication of diabetes and the leading cause of ESRD in industrialized countries.¹ Clinically, DN is manifested by microalbuminuria, proteinuria, and progressive glomerular dysfunction. The main pathologic features of DN include podocyte loss, mesangial cell hypertrophy, glomerular basement membrane thickening, glomerulosclerosis, and tubulointerstitial fibrosis.^{2–4} With the current

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standard therapies, including angiotensin-converting enzyme inhibitors and/or angiotensin receptor blockers, only partial renal protection is obtained.^{5–7} Thus, it is of particular importance to understand more about the pathogenesis of DN and to identify novel therapeutic targets in order to develop new therapies that will prevent or delay the progression of DN.

The endothelin (ET) system has recently emerged as an interesting novel target for the treatment of DN. ET-1 is a powerful mitogen and vasoconstrictor that influences a wide variety of organ functions and has been implicated in several cardiovascular and renal pathologies.^{8,9} ET-1 signals through two G protein-coupled receptors (GPCRs), endothelin receptor type A (ETAR) and endothelin receptor type B (ETBR), and can lead to the activation of a variety of signaling cascades such as NF- κ B,¹⁰ β -catenin, phosphoinositide 3-kinase, or mitogen-activated protein kinase.^{11–13} Both receptors are present in the kidney.^{14–17} It was early recognized that ET-1 displays proliferative effects on mesangial cells that are mediated by ETAR.^{18,19} At the glomerular level, ET-1 promotes mesangial cell proliferation, sclerosis, and podocyte injury, although it is not demonstrated whether this latter effect is direct.^{8,18,20}

Several lines of evidence suggest a specific role for the ET signaling pathway in the pathogenesis of DN. ET-1 expression is increased in kidneys with DN and higher ET-1 concentrations are found in the circulation of patients with DN as well as in animal models of DN.^{21–25} In diabetic db/db mice, mesangial matrix expansion was shown to be temporally and spatially associated with glomerular immunoreactivity for ET-1.²⁶ ET receptor (ETR) blockers have been shown to be nephroprotective in diabetic animals in a BP-independent manner.^{27–32} In patients with DN, results from clinical trials of ETR blockers depend on the general cardiovascular status of patients modulating tolerance to sodium and water retention as well as on the drug used.^{33,34} Nevertheless, recent clinical studies are encouraging and suggest that ETAR antagonists are not only capable of promoting a regression of proteinuria, but may also limit glomerulosclerosis-related renal injury.^{31,32,35} ETAR antagonists were found to have anti-inflammatory and antifibrotic effects during experimental DN,^{29,36} but the cell types that promote DN under the influence of ET-1 are still not known. Furthermore, despite the major role of podocyte dysfunction in DN, the specific involvement of ET-1 signaling in podocytes has not been fully investigated.⁸ Therefore, this study aimed to investigate the ET-1 signaling pathway in podocytes during diabetes-induced nephropathy.

In this study, we show that mice with a podocyte-specific double deletion of *Ednar* and *Ednbr* alleles are protected from diabetes-induced glomerulosclerosis and podocyte loss. We found the first evidence that ET-1 activation in the kidney drives development of diabetic glomerulosclerosis and podocyte loss with direct activation of ETRs and NF- κ B and β -catenin pathways in podocytes. Surprisingly, the ETBR subtype was found to be important both at the expression level and the functional level. *Ednbr* mRNA expression in primary podocytes was found to be two times higher than *Ednar*

expression, and a ETB agonist elicited calcium mobilization with β -catenin and NF- κ B signaling. Furthermore, these results indicate that selective activation of the ETR pathways in podocytes is involved in pathophysiologic cellular crosstalk that influences mesangial architecture and sclerosis.

RESULTS

Characterization of the Podocyte-Specific ETAR/ETBR Knockout Mice

To determine whether the ET pathway may be involved in podocyte pathogenesis during DN, we generated mice with a podocyte-specific double deletion of *Ednar* and *Ednbr* by using the NPHS2-Cre recombinase (Pod-Cre), which expresses Cre recombinase exclusively in podocytes starting from the capillary loop stage during glomerular development.³⁷ This was confirmed by RT-PCR of *Ednar* and *Ednbr* mRNA. RT-PCR showed a statistically significant reduction in *Ednar* and *Ednbr* mRNA levels in isolated podocytes of NPHS2-Cre *Ednar*^{lox/lox} *Ednbr*^{lox/lox} (Pod-ETRKO) mice compared with control wild-type (WT) mice (Figure 1A). The purity of primary podocyte culture was validated by nephrin and podocin immunostaining (Supplemental Figure 1). Likewise, Western blot analyses of ETBR expression showed a significant decrease in ETBR expression (approximately 64%) in glomeruli from Pod-ETRKO mice (Figure 1B), suggesting that ETBR expression in podocytes accounts for the majority of ETBR in normal glomeruli. Pod-ETRKO mice developed normally until at least 12 months and had no gross morphologic and physiologic abnormalities (data not shown) (Figure 1C, Table 1). Renal function of Pod-ETRKO mice in the basal state was not altered, as determined by urinary albumin excretion and BUN levels (Table 1). Histologic and electronic microscopy analyses of glomeruli did not reveal any abnormality in glomerular ultrastructure from Pod-ETRKO mice compared with WT mice at basal state (Figure 1, C and D).

Endothelin Signaling in Podocytes Is Mediated Mainly by ETBR

We analyzed ET-1 signaling in primary mouse podocytes by measuring the variations of intracellular Ca²⁺ in cultured podocytes after stimulation with thrombin as a positive control or ET-1 and demonstrated functional receptors as ET-1 elicited rapid calcium transients (Figure 2). The ETBR-selective agonist sarafotoxin 6c induced a maximal calcium response similar to the one elicited by ET-1, thus showing that ETBR is functionally important in primary podocytes (Figure 2). Furthermore, the ETAR antagonist, BQ-123, as well as the ETBR antagonist, BQ-788, induced an approximately 65% decrease in ET-1-induced calcium transient, whereas a combination of both antagonists synergized this effect (Supplemental Figure 2). Taken together, these results show that both ETAR and ETBR are functional in podocytes, mediating ET-1-induced calcium release.

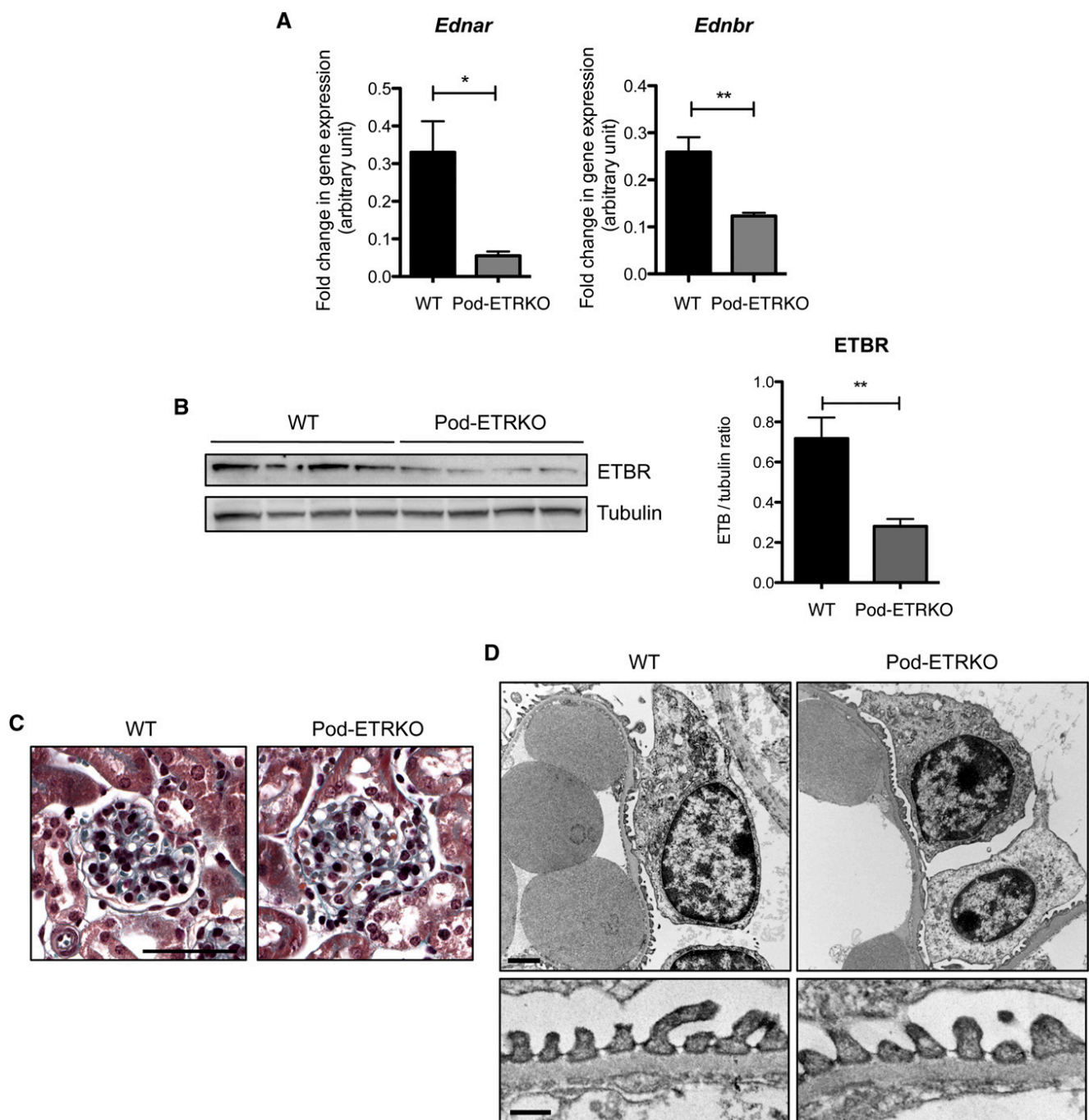


Figure 1. ETAR and ETBR podocyte-specific deletions do not alter glomerular structure. (A) RT-PCR analysis of *Ednar* and *Ednbr* mRNA expression in isolated podocytes from 10-week-old WT and Pod-ETRKO mice. (B) Effective deletion of ETBR protein by NPHS2-Cre recombinase confirmed by immunoblotting analysis of isolated glomerular homogenates. Quantification of Western blot bands for ETBR normalized to tubulin band intensity. (C) Representative images of Masson's trichrome-stained sections of glomeruli from 10-week-old WT and Pod-ETRKO mice. (D) Representative photomicrograph of transmission electron microscopy sections of podocytes from 10-week-old WT and Pod-ETRKO mice. Values are the mean \pm SEM from four mice. * $P < 0.05$; ** $P < 0.01$. Scale bar, 50 μ m in C; 1 μ m in upper panel in D; 200 nm in lower panel in D.

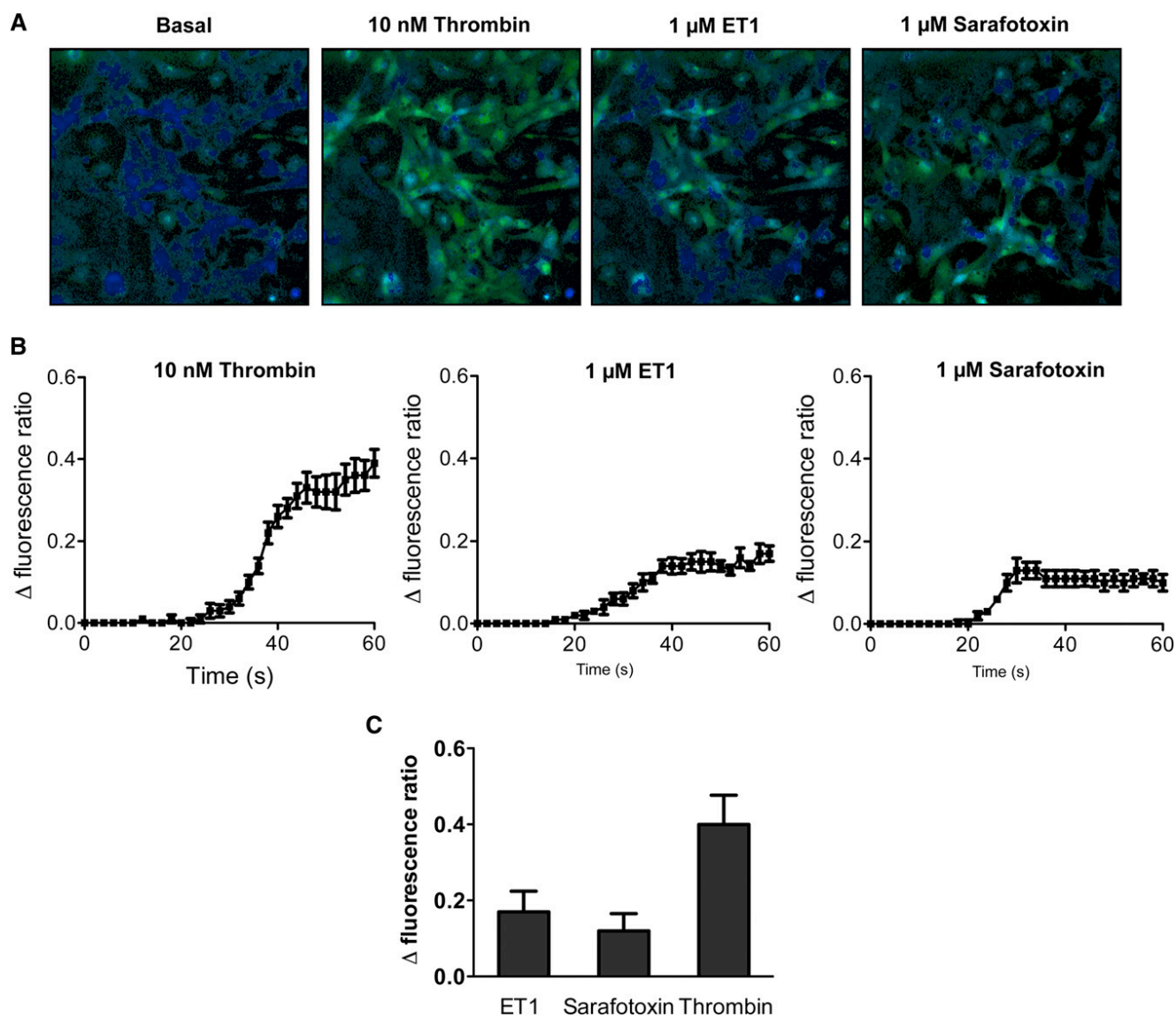
β -Catenin and NF- κ B Pathways Are Downregulated in Podocytes from Pod-ETRKO Mice

We next investigated which signaling pathways could be activated downstream to the ETR in podocytes. Because

ETAR and ETBR can activate β -catenin and NF- κ B pathways in nonglomerular cells,^{10,38–41} we analyzed the activation status of these pathways in glomeruli from Pod-ETRKO mice. We found that total β -catenin and phospho-NF κ B expressions

Table 1. Animal phenotype

| Parameter | WT Mice | | Pod-ETRKO Mice | |
|---|---------------|-------------------------|----------------|---------------------------|
| | Control (n=9) | Diabetes (n=15) | Control (n=9) | Diabetes (n=15) |
| Blood glucose (mg/dl) | 196.3±16.4 | 547.4±29.6 ^a | 171.4±7.6 | 537.6±17.4 ^{a,b} |
| Body weight (g) | 32.1±1.9 | 25.3±1.2 ^a | 29.8±1.3 | 24.2±1.5 ^{a,b} |
| Kidney to body weight ratio (%) | 0.60±0.02 | 0.84±0.03 ^a | 0.59±0.01 | 0.86±0.02 ^{a,b} |
| Urinary albumin to creatinine ratio (mg/mmol) | 0.54±0.14 | 3.19±0.60 ^a | 0.73±0.11 | 1.52±0.39 ^c |
| BUN (mmol/L) | 21.1±1.7 | 27.3±1.6 ^a | 22.1±0.6 | 29.2±2 ^{a,b} |

^aP<0.05 versus respective nondiabetic group with same genotype.^bP<0.05 versus WT nondiabetic mice.^cP<0.05 versus diabetic WT mice.**Figure 2.** Activation of the Ca²⁺ pathway in podocytes stimulated by thrombin, ET-1, and sarafotoxin 6c. (A) Typical cell images illustrating the change in fluorescence ratio induced after addition of ET-1 (1 μM), sarafotoxin 6c (1 μM), or thrombin (10 nM) compared with the basal fluorescence ratio. (B) Time course of the averaged responses to the compounds. (C) Summary of the maximal fluorescence ratios. Values are the mean±SEM of nine values representing three different experiments each performed in triplicate.

are reduced in glomeruli from Pod-ETRKO mice compared with WT mice in the basal state (Figure 3, A and B). β -Catenin and phospho-NF- κ B expression levels are also diminished in primary podocyte cultures from Pod-ETRKO mice compared with WT mice (Supplemental Figure 3). Moreover, we analyzed by RT-PCR the mRNA expression of the β -catenin target genes, *snai1*, *vimentin*, and *axin2* and found that these three genes are also downregulated in glomeruli from Pod-ETRKO mice in the basal state (Figure 3C).

ET-1 Activates Glomerular β -Catenin and NF- κ B Signaling Pathways *In Vitro*

We then sought to determine whether ET-1 could directly activate β -catenin and NF- κ B pathways in podocytes. Glomeruli from WT and Pod-ETRKO mice were treated with ET-1 (100 nM) for 4 hours, after which we analyzed the activation status of β -catenin and NF- κ B signaling pathways. Immunoblot analyses showed an increase in total β -catenin and phospho-NF- κ B protein expression in glomeruli from WT mice treated with ET-1 (Figure 4, A and B). Interestingly, baseline as well as ET-1-stimulated β -catenin and phospho-NF- κ B protein expressions in glomeruli from Pod-ETRKO

mice were significantly below levels in WT glomeruli (Figure 4, A and B). Moreover, maximum ET-1 stimulation of Pod-ETRKO glomeruli failed to trigger recruitment of β -catenin and phospho-NF- κ B above baseline levels of WT glomeruli (Figure 4, A and B). Involvement of β -catenin activity by podocyte ETRs was further confirmed because *snai1*, *vimentin*, and *axin2* mRNA expression induced by ET-1 in WT glomeruli could not be achieved in glomeruli from Pod-ETRKO mice (Figure 4C). Furthermore, selective ETBR stimulation with sarafotoxin 6c mimicked ET-1 actions on β -catenin and NF- κ B pathway activation (Supplemental Figure 4).

Podocyte-Specific Deletion of *Ednar* and *Ednrb* Protected Mice from Diabetes-Induced Glomerulosclerosis

We then examined the role of the ET pathway in podocytes after diabetes mellitus (DM) induction by streptozotocin injection. Ten weeks after diabetes induction, mice presented polyuria and weight loss (data not shown) (Table 1). Pod-ETRKO DM and WT DM mice developed features of mild DN, as determined by an increased kidney/body weight ratio and albuminuria (Table 1). WT DM mice developed a higher urinary

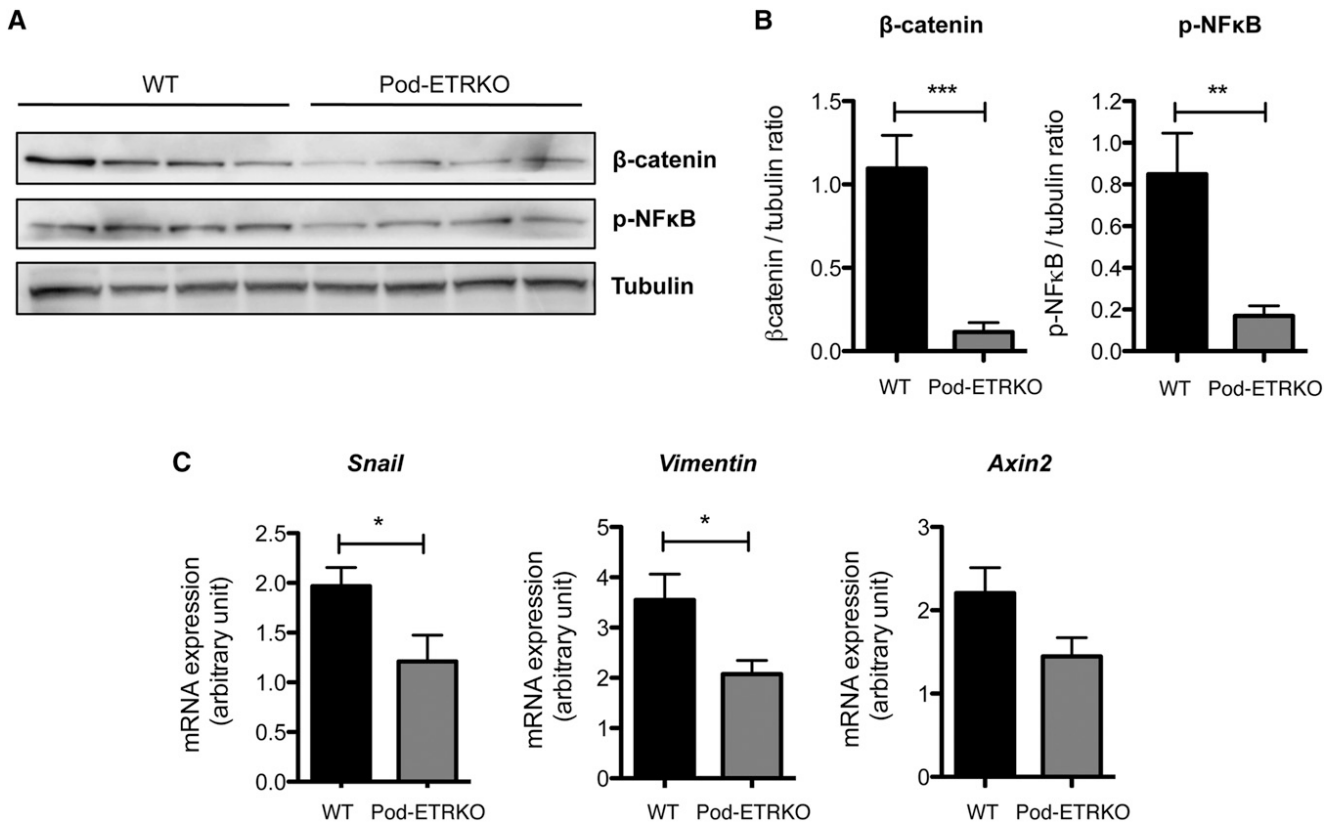


Figure 3. At basal state, β -catenin and NF- κ B signaling pathways are decreased in glomeruli of Pod-ETRKO mice. (A) Western blot analysis of β -catenin and phospho-NF- κ B (p-NF- κ B) expression in glomerular extracts from 10-week-old WT and Pod-ETRKO mice. Protein concentration is normalized to tubulin expression. (B) Quantification of Western blot bands for β -catenin and p-NF- κ B normalized to tubulin band intensity. (C) RT-PCR analysis of *Snai1*, *Vimentin*, and *Axin2* mRNA expression in glomerular extracts from 10-week-old WT and Pod-ETRKO mice. Values are the mean \pm SEM from at least six mice. * P <0.05; ** P <0.01; *** P <0.001.

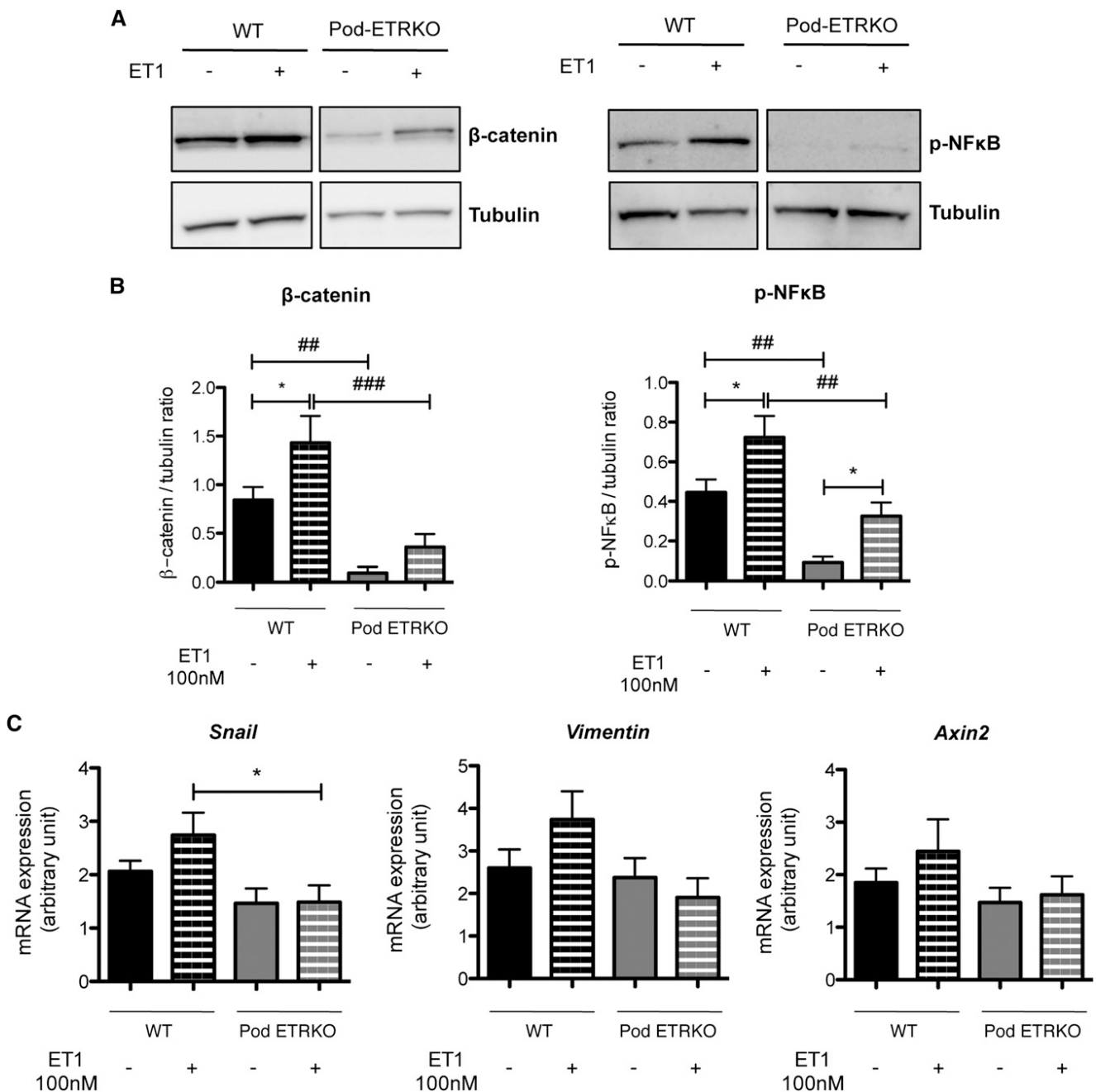


Figure 4. *In vitro*, endothelin-1 activates β -catenin and NF- κ B signaling pathways in glomeruli and its induction is decreased in Pod-ETRKO mice. (A) Western blot analysis of β -catenin and phospho-NF- κ B (p-NF- κ B) expression in glomeruli extracts from 10-week-old WT and Pod-ETRKO mice, treated or not with ET-1 at 100 nM for 4 hours. Protein concentration is normalized to tubulin expression. (B) Quantification of Western blot bands for β -catenin and p-NF- κ B normalized to tubulin band intensity. (C) RT-PCR analysis of *Snai1*, *Vimentin*, and *Axin2* mRNA expression in glomerular extracts from 10-week-old WT and Pod-ETRKO mice, treated or not with ET-1 at 100 nM for 4 hours. Values are the mean \pm SEM from at least six mice. * P <0.05 versus respective non-ET-1-treated mice; ## P <0.01 versus respective WT mice; ### P <0.001 versus respective WT mice.

albumin excretion rate than Pod-ETRKO DM mice ($P=0.03$ for WT DM versus Pod-ETRKO DM) (Table 1). Histologic analyses showed tubular dilations and glomerular capillary dilations in the kidney of both WT diabetic and Pod-ETRKO diabetic mice (Figure 5, A and B). We analyzed the histologic

structure of the glomeruli from WT and Pod-ETRKO diabetic mice. More than half of the glomeruli from WT diabetic mice displayed mesangial thickening (approximately 58%), whereas glomeruli from Pod-ETRKO diabetic mice presented no such feature of glomerulosclerosis (Figure 5, B–D). This

result was confirmed by electron microscopy (Supplemental Figure 5). Notably, *preproendothelin-1* (*Edn1*) mRNA expression was similarly upregulated in glomeruli from diabetic WT and Pod-ETRKO mice (Figure 5E). We next analyzed the expression of two genes associated with sclerosis, *Ctgf* and *Trpc6*, and found that these two genes were significantly more upregulated in glomeruli from WT diabetic mice (+138% for *Ctgf* and +124% for *Trpc6*, WT DM versus WT) than in glomeruli from Pod-ETRKO diabetic mice (+43% for *Ctgf* and +37% for *Trpc6*, Pod-ETRKO DM versus Pod-ETRKO) (Figure 5E).

Podocyte-Specific Deletion of *Ednar* and *Ednbr* Protected Mice from Diabetes-Induced Podocytopathy

We next sought to investigate podocyte structure and number in diabetic mice. Podocalyxin and podocin staining showed weaker immunofluorescence in glomeruli from WT diabetic mice than in nondiabetic WT animals, thus demonstrating alterations in podocyte differentiation with diabetes. Podocalyxin and podocin immunostainings were strong and of similar intensity and pattern in WT and Pod-ETRKO nondiabetic mice. Diabetic Pod-ETRKO mice showed intermediate podocalyxin and podocin staining intensity, suggesting that podocyte alterations are less important in Pod-ETRKO diabetic mice (Figure 6A). Podocyte number per glomerulus, as determined by Wilms' tumor antigen 1 (WT1) immunohistochemistry, was significantly decreased in WT diabetic mice (−19% WT versus WT DM), whereas podocyte number in Pod-ETRKO diabetic kidneys remained similar to that measured in nondiabetic WT and Pod-ETRKO kidneys (Figure 6, B and C). Finally, electron microscopy analyses showed glomerular basement membrane thickening and podocyte foot process effacement in WT diabetic mice, whereas few ultrastructural defects were found in podocytes from Pod-ETRKO diabetic mice (Figure 6D).

We then examined the role of the ET pathway in podocytes in a model of streptozotocin-induced diabetes after uninephrectomy, a more severe model of DN. Similar alleviation of glomerular damage was consistently found in Pod-ETRKO diabetic mice after uninephrectomy (Pod-ETRKO Nx+DM) compared with uninephrectomized WT diabetic mice (WT Nx+DM), with less glomerulosclerosis (Figure 7A) and maintenance of podocyte number (Figure 7, B and C).

Upon Diabetes, the β -Catenin Pathway Is Activated in Glomeruli by Podocyte ETRs

Because activation of the β -catenin pathways in glomeruli from diabetic mice was recently described,^{42,43} we examined the expression of the β -catenin target genes, *snail*, *vimentin*, and *axin2*, in glomeruli from WT and Pod-ETRKO diabetic mice. *Snail*, *vimentin*, and *axin2* mRNA expression was significantly increased in glomeruli from diabetic WT mice compared with glomeruli from control WT mice: +347% for *snail*, +202% for *vimentin*, and +236% for *axin2* (Figure 8). *Snail*, *vimentin*, and *axin2* mRNA expression was less induced in Pod-ETRKO DM glomeruli (+169% for *Snail*, +159% for

Vimentin, and +147% for *Axin2*, Pod-ETRKO DM versus Pod-ETRKO). *Snail* and *Axin2* mRNA glomerular expression was significantly lower in glomeruli isolated from diabetic mice with selective ETR gene deletions compared with normal glomeruli ($P < 0.05$), suggesting that podocyte ETRs significantly contribute to diabetes-induced activation of the β -catenin cascade (Figure 8).

DISCUSSION

In this study, we demonstrate that in streptozotocin-induced diabetes, mice with a podocyte-specific deletion of both *Ednar* and *Ednbr* (Pod-ETRKO) are protected from diabetes-induced glomerulosclerosis and podocyte loss, thus showing that ET signaling activation in podocytes increases susceptibility to develop DN. These results shed light on the pathophysiologic actions of the ET-1 system in DN and represent an example of the involvement of podocyte GPCRs in the pathophysiology of DN.

ET-1 activation in kidney tubules and glomeruli as well as in blood during DN is now well established,^{21–23} but the targeted cells of ET-1 and the mechanisms connecting ET-1 activation with the development of DN and particularly with podocytopathy development are still not completely elucidated. Sasser *et al.* demonstrated that the ETAR antagonist ABT-627 reduced diabetes-induced macrophage infiltration and TGF- β urinary excretion.²⁹ In this study, the authors suggest that ETAR activation in macrophages by increased renal production of ET-1 leads to macrophage recruitment in the kidney and TGF- β production by macrophages. Moreover, it has been shown that high glucose levels induce mesangial cell ET-1 production⁴⁴ and that ET-1 promotes mesangial cell contraction, hypertrophy, and generation of inflammatory mediators, cytokines, and growth factors as well as the production of fibronectin *in vitro*. On the other hand, little is known about the mechanisms through which ET-1 might directly affect podocyte cell survival and/or function, particularly during diabetes. Disruption of the actin cytoskeleton has been observed after *in vitro* exposure to ET-1 or toxic compounds such as puromycin aminonucleoside or Shiga toxin in an ETAR-dependent manner in a mouse immortalized podocyte line.^{45–47} Meanwhile, expression (or lack of) of the ETBR was not searched for in this immortalized cell line. We observed high ETBR expression in primary culture of podocytes and in freshly isolated glomeruli as reported in a seminal article in rat glomeruli.¹⁶ ETBR expression in podocytes was indeed described by immunoelectron microscopy that allowed visualization of intense immunodeposits indicating ETBR in fully differentiated rat podocytes *in situ*, particularly in their foot processes.¹⁶ Other researchers found immunoreactive ETAR and ETBR in mesangial and glomerular capillary cells, respectively¹⁷; variable staining for immunoreactive ETBR in glomeruli (in parietal epithelial cells and podocytes) has been reported in kidney biopsies from healthy controls and from patients with

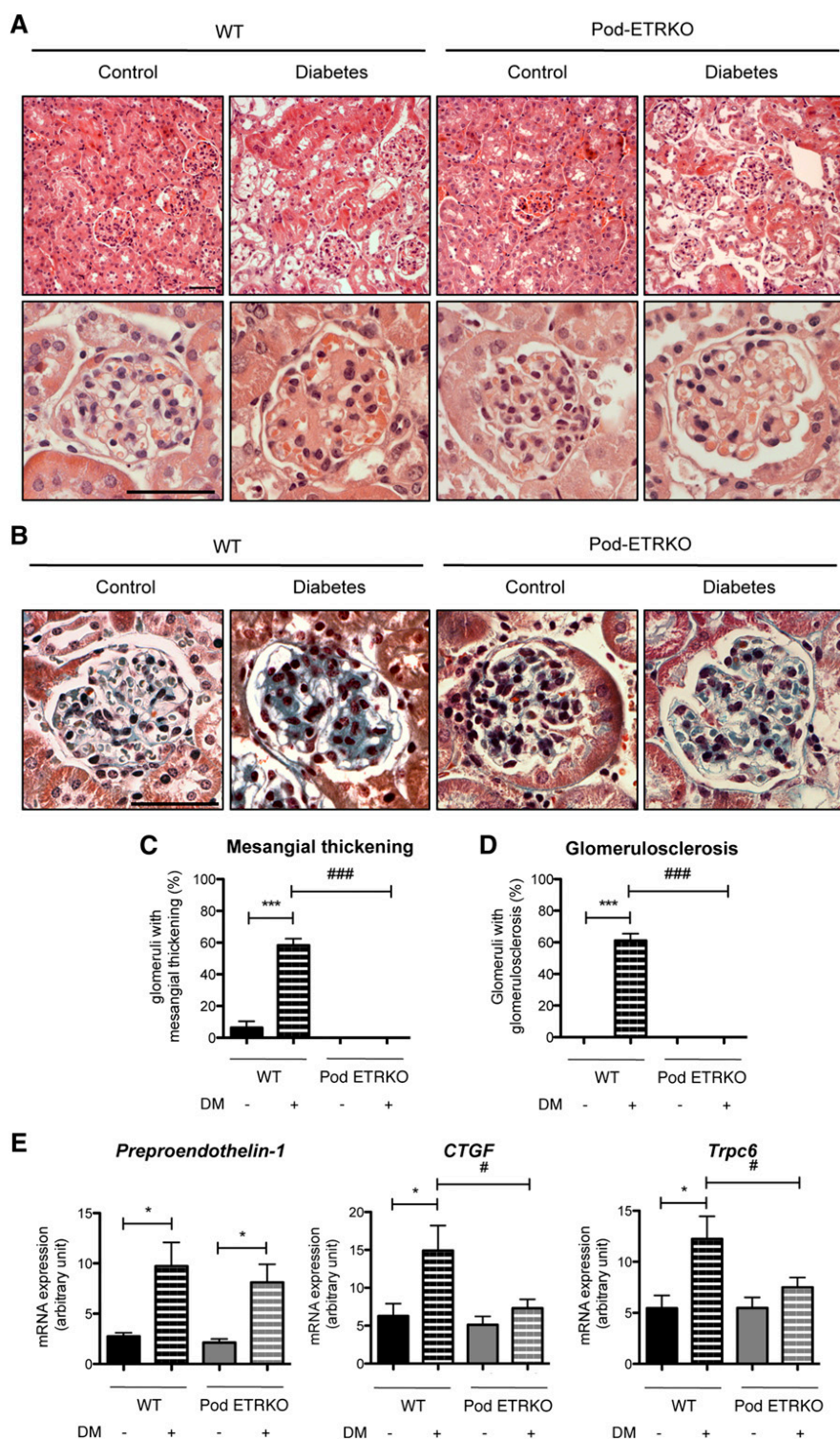


Figure 5. Deletions of *Ednar* and *Ednbr* specifically in podocytes protect glomeruli from diabetes-induced glomerulosclerosis. (A) Representative images of hematoxylin/eosin-stained sections of renal cortex from 20-week-old WT control, WT diabetic, Pod-ETRKO control, and Pod-ETRKO diabetic mice. (B) Representative images of Masson's trichrome-stained sections of glomeruli from 20-week-old WT control, WT diabetic, Pod-ETRKO control, and Pod-ETRKO diabetic mice. (C and D) Percentage of glomeruli with mesangial thickening (C) in the renal cortex of 20-week-old WT control, WT diabetic, Pod-ETRKO control, and Pod-ETRKO diabetic mice. (D) RT-PCR analysis

IgA nephropathy,¹⁴ confirming the findings reported by Karet *et al.*¹⁵ Here we detected ETAR and ETBR expression in primary podocytes, along with novel functional data. ET-1 stimulates calcium transients in podocytes in an ETAR- as well as ETBR-dependent fashion. This intriguing observation suggests that ETAR and ETBR may cosignal, potentially as heterodimers as recently suggested.^{48–50}

For the first time, we further observed that sarafotoxin 6c, a selective agonist of ETBR, was sufficient to activate calcium and extracellular signal-regulated kinase signaling, thus showing that the ETBR signaling is functionally significant in primary podocytes. Moreover, sarafotoxin 6c stimulation in freshly isolated glomeruli also activated NF- κ B and β -catenin, thus suggesting that chronic NF- κ B and β -catenin activation in diabetic glomeruli is mediated at least in part by ETBR.

Surprisingly, therapy for CKD has focused on ETAR antagonists only, based on previous experimental findings indicating that ETAR promotes mesangial sclerosis and glomerular capillary pressure.^{8,29,51–53} Indeed, the partial benefits of ETAR antagonists for the treatment of DN have been extensively discussed after clinical data revealed that both ETAR and ETBR promote some degree of fluid retention in patients at an advanced stage.^{33,34,54} Here we point out that the combination of ETAR antagonists with ETBR antagonists may provide additional beneficial effects on glomerular remodeling and function in diabetes. In line with our mechanistic findings, recent clinical data indicate that dual endothelin receptor antagonism improves peripheral endothelial function in patients with type 2 diabetes and microalbuminuria.⁵⁵

of *preproendothelin-1* (*Edn1* gene), *Ctgf*, and *trpc6* mRNA expression in glomerular extracts from 20-week-old WT control, WT diabetic, Pod-ETRKO control, and Pod-ETRKO diabetic mice. Values are the mean \pm SEM from at least six mice. * $P < 0.05$ versus respective non-diabetic mice; # $P < 0.05$ versus WT diabetic mice; *** $P < 0.001$ versus respective non-diabetic mice; ### $P < 0.001$ versus WT diabetic mice. Scale bar, 50 μ m.

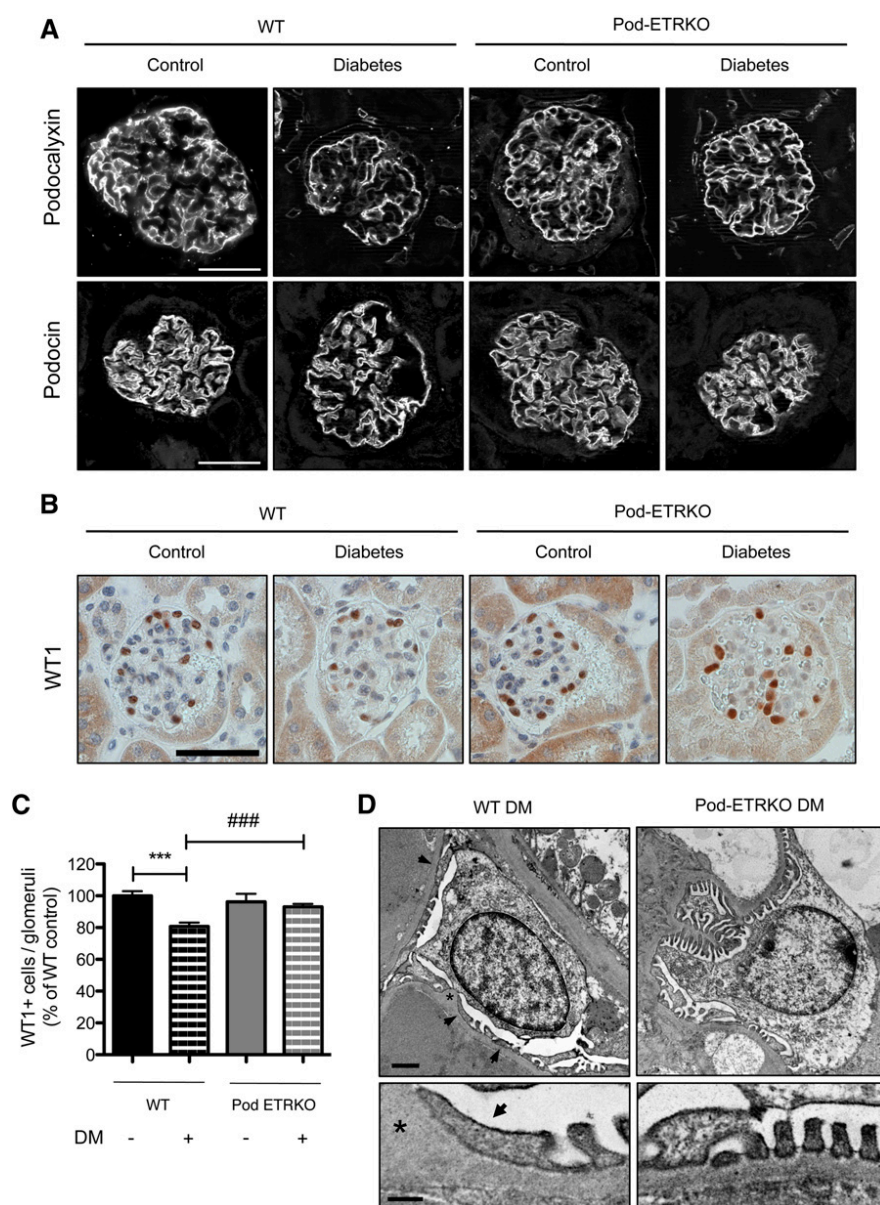


Figure 6. ETAR and ETBR podocyte-specific deficiency protects podocytes from diabetes-induced podocyte loss. (A) Representative images of the expression of podocalyxin (upper panel) and podocin (lower panel) by immunofluorescence in 20-week-old WT control, WT diabetic, Pod-ETRKO control, and Pod-ETRKO diabetic mice. Images are representative of at least six mice. (B) Representative images of the expression of WT1 by immunohistochemistry in 20-week-old WT control, WT diabetic, Pod-ETRKO control, and Pod-ETRKO diabetic mice. Images are representative of at least six mice. (C) Quantification of the glomerular WT1-positive cell numbers in 20-week-old WT control, WT diabetic, Pod-ETRKO control, and Pod-ETRKO diabetic mice. Data are normalized to WT control and represent the mean \pm SEM from at least six mice. (D) Representative photomicrographs of transmission electron microscopy sections of podocytes from 20-week-old WT diabetic and Pod-ETRKO diabetic mice showing glomerular basement membrane thickening (asterisk) and foot process effacement (arrow) in WT DM mice. $***P < 0.001$ versus respective nondiabetic mice; $###P < 0.001$ versus WT diabetic mice. Scale bar, 50 μ m in A and B; 1 μ m in upper panel in D; 200 nm in lower panel in D.

We also identified that NF- κ B and β -catenin are activated downstream of the ETR after ET-1 stimulation in cultured glomeruli. Moreover, ET-1 and β -catenin pathways are activated in glomeruli during progression of DN. Although glomerular *prepro-ET-1* mRNA is overexpressed in Pod-ETRKO mice, β -catenin pathway activation does not still occur. Taken together, these results strongly suggest that ET-1 signaling is activated in podocytes during diabetes and it exerts its detrimental remodeling effects through activation of NF- κ B and β -catenin pathways. It is noteworthy that Pod-ETRKO glomeruli stimulated with exogenous ET-1 showed reduced but still residual activation of NF- κ B and β -catenin pathways, suggesting that residual activation of NF- κ B and β -catenin pathways elicited by ET-1 may have occurred in other glomerular cell types. It is well established that ET-1 can drive NF- κ B and β -catenin pathway activation in several cell types; however, no information is available thus far regarding the downstream effectors of the ETR in podocytes.^{10,38–41} NF- κ B was recently implicated in glomerular aging, by promoting inflammation, coagulation, and fibrosis.^{56,57} A delicate balance of β -catenin expression in podocytes was recently demonstrated to be critical for podocyte cell adhesion and survival.⁴³ β -catenin overexpression in podocytes during DN or FSGS promoted podocyte dysfunction and albuminuria, whereas blockade of the β -catenin pathway ameliorated kidney injury.^{42,43} Here we provide evidence that ETR activation in podocytes during diabetes is a potent upstream event leading to NF- κ B and β -catenin activation and podocytopathy.

Surprisingly, we found that podocyte deletion of ETR protects not only from diabetes-induced loss of podocytes but also from mesangial matrix expansion. Recent studies indicated that isolated podocyte damage leads to glomerulosclerosis⁵⁸ and that podocyte loss appears to be a requisite early event in DN.⁵⁹ Accordingly, podocyte loss in individuals with type 2 diabetes predicts nephropathy as well as mesangial expansion.⁶⁰ Presumably, signals from damaged podocytes to the mesangium, or

the hemodynamic factors triggered by podocyte loss, could provide a stimulus to the mesangial cell to react by increases

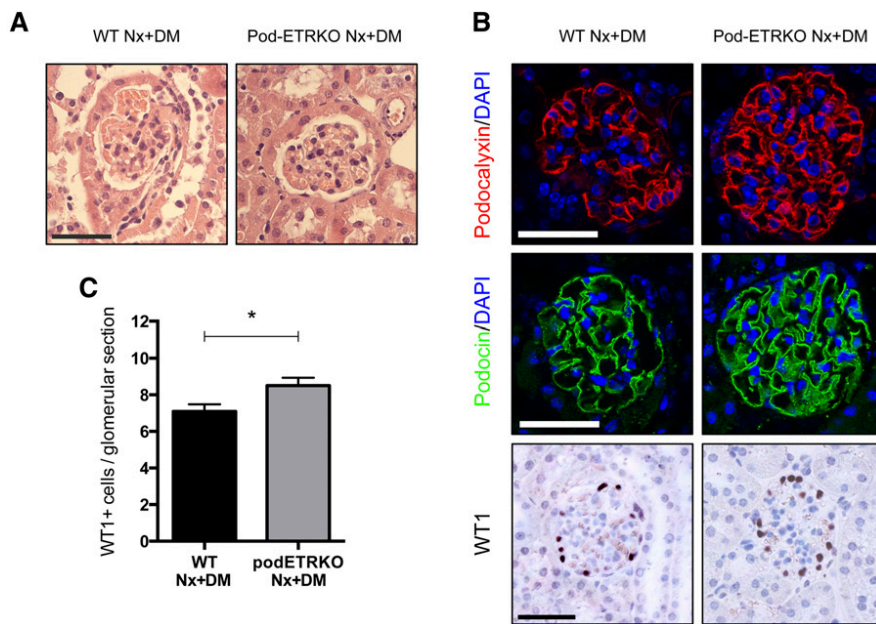


Figure 7. Pod-ETRKO mice are protected from diabetes-induced glomerulosclerosis and podocyte loss in a model of uninephrectomy followed by streptozotocin-diabetes induction. (A) Representative images of hematoxylin/eosin-stained sections of renal cortex from 16-week-old WT uninephrectomized diabetic and Pod-ETRKO uninephrectomized diabetic mice. (B) Representative images of the expression of podocalyxin (upper panel), podocin (middle panel), and WT1 (lower panel) in 16-week-old WT uninephrectomized diabetic and Pod-ETRKO uninephrectomized diabetic mice. (C) Quantification of the glomerular WT1-positive cell numbers in 16-week-old WT uninephrectomized diabetic and Pod-ETRKO uninephrectomized diabetic mice. Data represent the mean \pm SEM from at least four mice. * $P < 0.05$. DAPI, 4',6-diamidino-2-phenylindole. Scale bar, 50 μ m.

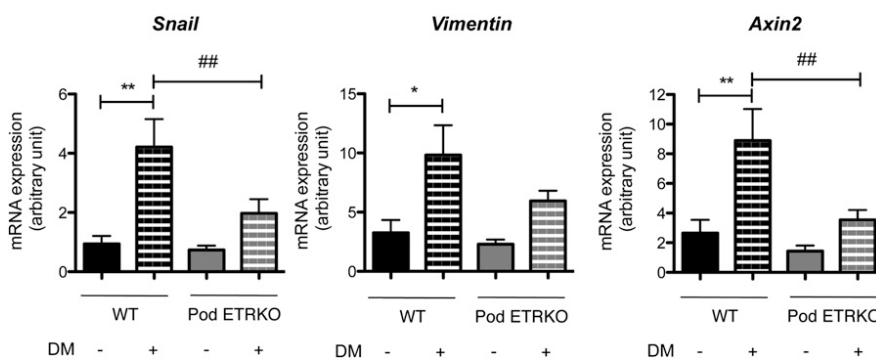


Figure 8. The induction of β -catenin and NF- κ B signaling pathways in diabetic glomeruli is limited in Pod-ETRKO glomeruli. RT-PCR analysis of *Snai1*, *Vimentin*, and *Axin2* mRNA expression in glomerular extracts from 20-week-old WT control, WT diabetic, Pod-ETRKO control, and Pod-ETRKO diabetic mice. Values are the mean \pm SEM from at least six mice. * $P < 0.05$ versus respective nondiabetic mice; ** $P < 0.01$ versus respective nondiabetic mice; ## $P < 0.01$ versus WT diabetic mice.

in extracellular matrix synthesis or decreases in extracellular matrix degradation. Our data suggest that modulation of the ET-1 pathway in podocytes influenced crosstalk between podocytes and mesangial cells. Indeed, it was demonstrated that

cell culture medium conditioned by podocytes induced mesangial cell proliferation *in vitro*.⁶¹ Moreover, podocyte connective tissue growth factor (CTGF) seems to have paracrine effects on mesangial cells to stimulate CTGF expression.⁶² Interestingly, diabetic normal mice displayed a 3-fold increase in *Ctgf* glomerular mRNA expression compared with their Pod-ETRKO counterparts. These results are compatible with the hypothesis that ETRs stimulate a paracrine factor secreted by podocytes, such as CTGF, that in turn may promote mesangial cells lesions during diabetes. Taken together, these studies identify the first GPCRs in podocytes that mediate progression of DN and support the notion that persistent ETAR and ETBR activation in podocytes is sufficient to promote glomerular injury with podocyte depletion through recruitment of the β -catenin pathway.

CONCISE METHODS

Animals

Mice with podocyte-specific disruption of both *Ednar* and *Ednbr* genes (podocin-Cre⁺ *Ednar*^{lox/lox} *Ednbr*^{lox/lox} mice) were generated by crossing NPHS2-CRE⁺ mice³⁷ with *Ednar*^{lox/lox63} and *Ednbr*^{lox/lox64} mice. Mice are on a mixed C57BL/6J/129/SV/FVB/N genetic background. Animals homozygous for floxed *Ednar* and *Ednbr* genes but without Cre were used as controls in all studies. For animals subjected to left uninephrectomy, animals were anesthetized by isoflurane inhalation and received 0.1 mg/kg buprenorphine twice daily for 2 days. The left renal artery and vein were ligated with 6-0 silk before kidney excision and the wound was closed in layers. Diabetes was induced after 7 days of recovery. All mice were given free access to water and standard chow. Experiments were conducted according to the French veterinary guidelines and those formulated by the European Commission for experimental animal use (L358-86/609EEC) and were approved by the Institut National de la Santé et de la Recherche Médicale (INSERM).

Induction of DM with Streptozotocin

Twelve-week-old male mice were rendered diabetic with streptozotocin (100 mg/kg in citrate buffer, pH 4.5) by intraperitoneal injection on 2 consecutive days. Control mice received citrate buffer alone. Mice with fasted glycemia > 300 mg/dl were considered diabetic. Mice were euthanized 10 weeks after diabetes

induction and uninephrectomized diabetic mice were euthanized 4 weeks after diabetes induction.

Assessment of Renal Function and Albuminuria

Urinary creatinine and BUN concentrations were quantified spectrophotometrically using colorimetric methods. Urinary albumin excretion was measured using a specific ELISA assay for quantitative determination of albumin in mouse urine (BIOTREND Chemikalien GmbH).

Isolation of Glomerular and Podocyte Cultures

Decapsulated glomeruli were isolated as previously described.^{65,66} Briefly, freshly isolated renal cortex was mixed and digested by collagenase I (2 mg/ml; Gibco) in RPMI 1640 (Life Technologies) for 2 minutes at 37°C, and then collagenase I was inactivated with RPMI 1640 plus 10% FCS (Abcys). Tissues were then passed through a 70- μ m cell strainer and a 40- μ m cell strainer (BD Falcon) in PBS (Life Technologies) plus 0.5% BSA (Sigma-Aldrich). Glomeruli, adherent to the 40- μ m cell strainer, were removed from the cell strainer with PBS plus 0.5% BSA injected under pressure, and then washed twice in PBS. Isolated glomeruli were then picked up in Phosphosafe extraction buffer (Novagen) and frozen at -80°C for protein extraction, or picked up in RLT extraction buffer (Qiagen) and frozen at -80°C for total RNA extraction or then processed for cell culture. For podocyte primary cell culture, freshly isolated glomeruli were plated in six-well dishes in RPMI 1640 plus 10% FCS and 1% penicillin/streptomycin (Life Technologies). Two days after seeding, glomeruli became adherent to the dish, and podocytes exited from glomeruli and started growing in the dish. Isolated podocytes were picked up in Phosphosafe extraction buffer and frozen at -80°C for protein extraction or picked up in RLT extraction buffer and frozen at -80°C for total RNA extraction.

Intracellular Calcium Measurements

Variations of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) were measured in cultured podocytes using the ratiometric fluorescence Ca^{2+} indicator Fura-2 as previously described.⁶⁷ Cells subcultured for 4 days in chambered coverglass (Lab-Tek) were loaded by incubation with 2.5 mmol/L Fura-2AM (TEFLabs) plus 0.02% pluronic acid F-127 (Molecular Probes, Inc.), in Locke buffer for 20 minutes at 37°C in a humidified air atmosphere. Cells were rinsed with buffer and mounted on a microscope stage (Axiovert, $\times 20$ objective; Zeiss) during a 15-minute waiting period for the de-esterification of Fura-2AM. Media and drugs (ET-1, sarafotoxin 6c, and thrombin) were then applied to the cells using a perfusion system. Images were captured digitally every 2 seconds with a cooled charge-coupled device camera (Photometrics, Roper Scientific). Cells were illuminated by excitation with a dual ultraviolet light source at 340 nm (Ca^{2+} -bound) and 380 nm (Ca^{2+} -free) using a Lambda DG-4 excitation system (Sutter Instrument Company). Fluorescence emission was measured at 510 nm and analyzed using MetaFluor software (Universal Imaging Corporation). Changes in $[\text{Ca}^{2+}]_i$ were deduced from variations of the F340/F380 ratio after correction for background and dark currents. Data were averaged with n representing the number of fields (at least 100 cells per recorded field).

Histopathology and Immunohistochemistry

Kidneys were immersed in 10% formalin and embedded in paraffin. Sections (4- μ m thick) were processed for histopathology study or immunohistochemistry. Sections were stained with hematoxylin and eosin or Masson's trichrome stain. The proportion of pathologic glomeruli was evaluated by examination of at least 50 glomeruli per section by an examiner (P.-L.T.) who was blinded to the experimental conditions. For immunohistochemistry, we stained paraffin-embedded sections with the following primary antibodies: rabbit anti-WT1 (1:400; Santa Cruz Biotechnology, Inc.) and goat anti-podocalyxin (1:200; R&D Systems). For WT1 staining, specific staining was revealed using Histofine reagents (Nichirei Biosciences). For podocin immunofluorescence, fresh cryostat sections (4- μ m thick) were immediately fixed in 4% paraformaldehyde and then incubated with a goat anti-podocin antibody (1:100; Santa Cruz Biotechnology, Inc.). For podocalyxin and podocin immunofluorescence, we used a secondary rabbit anti-goat IgG AF594-conjugated antibody (1:400; Invitrogen). The nuclei were stained using 4',6-diamidino-2-phenylindole. Photomicrographs were performed with an Axiophot Zeiss photomicroscope.

Electron Microscopy

Small pieces of renal cortex were fixed in 2% glutaraldehyde, postfixed in 1% osmium tetroxide, and embedded in Araldite M (Sigma-Aldrich). Ultrathin sections were counterstained with uranyl acetate and lead citrate and examined using a transmission electron microscope.

Western Blot Analyses

Glomerular and podocyte lysates were prepared using Phosphosafe extraction buffer. Equal amounts of proteins were loaded on SDS-PAGE gels for separation and transferred onto polyvinylidene difluoride membranes. After blocking with milk, the membranes were probed with the following antibodies: rabbit anti-ETBR (1:1000; Santa Cruz Biotechnology, Inc.), rabbit anti- β -catenin (1:1000; Cell Signaling Technologies), rabbit anti-phospho-NF- κ B (p65 Ser536, 1:1000; Cell Signaling Technologies), and rat anti-tubulin (1:5000; Abcam). The results were visualized with horseradish peroxidase-conjugated secondary antibodies (1:2000; Cell Signaling Technologies) and enhanced chemiluminescence (Supersignal West Pico). The LAS-4000 imaging system (FujiFilm) was used to reveal bands and densitometric analysis was used for quantification.

Real-Time RT-PCR

Total RNA extraction of mice glomeruli was performed using an RNeasy Microkit (Qiagen) and reverse transcribed with the QuantiTect Reverse Transcription Kit (Qiagen). Maxima SYBR Green/Rox qPCR Mix (Thermoscientific Fermentas) was used to amplify cDNA for 40 cycles on an ABI PRISM thermocycler. The comparative method of relative quantification ($2^{-\Delta\Delta\text{CT}}$) was used to calculate the expression level of each target gene, normalized to glyceraldehyde 3-phosphate dehydrogenase. The oligonucleotide sequences are available upon request. Data are presented as the fold change in gene expression.

Statistical Analyses

Data are expressed as the mean \pm SEM. Statistical analyses were calculated using Prism 5.04 software (GraphPad Software, Inc.).

Between-group comparisons were performed using the two-tailed *t* test, whereas multiple-group comparisons between were performed using one-way ANOVA followed by the Newman–Keuls test. A *P* value <0.05 was considered statistically significant.

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DISCLOSURES

None.

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