CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

PTGER1 Deletion Attenuates Renal Injury in Diabetic Mouse Models

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We hypothesized that the EP1 receptor promotes renal damage in diabetic nephropathy. We rendered EP1 (*PTGER1*, official symbol) knockout mice (*EP1*^{-/-}) diabetic using the streptozotocin and OVE26 models. Albuminuria, mesangial matrix expansion, and glomerular hypertrophy were each blunted in *EP1*^{-/-} streptozotocin and OVE26 cohorts compared with wild-type counterparts. Although diabetes-associated podocyte depletion was unaffected by EP1 deletion, EP1 antagonism with ONO-8711 in cultured podocytes decreased angiotensin II-mediated superoxide generation, suggesting that EP1-associated injury of remaining podocytes *in vivo* could contribute to filtration barrier dysfunction. Accordingly, EP1 deletion in OVE26 mice prevented nephrin mRNA expression down-regulation and ameliorated glomerular basement membrane thickening and foot process effacement. Moreover, EP1 deletion reduced diabetes-induced expression of fibrotic markers fibronectin and α -actin, whereas EP1 antagonism decreased fibronectin in cultured proximal tubule cells. Similarly, proximal tubule megalin expression was reduced by diabetes but was preserved in *EP1*^{-/-} mice. Finally, the diabetes-associated increase in angiotensin II-mediated constriction of isolated mesenteric arteries was blunted in OVE26*EP1*^{-/-} mice, demonstrating a role for EP1 receptors in the diabetic vasculature. These data suggest that EP1 activation contributes to diabetic nephropathy progression at several locations, including podocytes, proximal tubule, and the vasculature. The EP1 receptor facilitates the actions of angiotensin II, thereby suggesting that targeting of both the renin-angiotensin system and the EP1 receptor could be beneficial in diabetic nephropathy. (*Am J Pathol* 2013, 183: 1789–1802; <http://dx.doi.org/10.1016/j.ajpath.2013.08.022>)

Accounting for nearly 40% of newly diagnosed cases of end-stage renal disease that require dialysis or renal transplantation and occurring in almost one-third of diabetes patients, diabetic nephropathy (DN) currently represents a major global health care burden.¹ Functional abnormalities commonly associated with DN include an early hyperfiltration phase followed by a declining glomerular filtration rate (GFR) and the onset of albuminuria, which can progress to overt proteinuria as disease worsens. Albuminuria is a hallmark clinical marker of DN and is an independent risk factor for the development of cardiovascular disease in diabetic and hypertensive patients.² Several factors contribute to DN, including but not limited to activated protein kinase C and mitogen activated protein kinase,³ increased profibrotic transforming growth factor (TGF)- β ,^{4,5} and an abnormal or overactive renin-angiotensin-aldosterone system

(RAAS).^{6–8} The latter is currently the main therapeutic target in DN because angiotensin II (AngII) type 1 (AT1) receptor blockers and angiotensin-converting enzyme inhibitors reduce albuminuria and preserve renal function in humans and rodents.^{9–12} However, because these agents only slow DN progression, the search for novel or complementary therapies is warranted.

Lipids are factors implicated in DN. Cyclooxygenases (COXs) catalyze the metabolism of arachidonic acid to unstable endoperoxide intermediates, which are then isomerized into prostanoids via tissue-specific synthases. Prostaglandin E₂ (PGE₂) is the most abundant renal prostanoid synthesized¹³ and exerts its effects through four distinct G protein-coupled

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E-type receptors (EP1 through EP4) encoded by *PTGER1-4* genes.^{14–17} PGE₂ acts in an autocrine/paracrine manner, promoting a variety of cell-signaling and physiologic responses, depending on local EP expression profile.¹⁸ COX-derived prostanoids are critical for regulation of salt excretion and blood pressure (BP) because a high-salt diet induces medullary COX-2 expression in mice.¹⁹ Moreover, COX-2 inhibition impairs renal sodium excretion, in part, by blunting PGE₂/EP2 activation in the collecting duct.^{20,21} However, in addition to its homeostatic function, COX-2 has been implicated in several diseases, including DN.²²

Renal COX-2 expression and prostaglandin levels are elevated in diabetes. Prostaglandin production is increased in isolated glomeruli and cultured mesangial cells derived from rats with streptozotocin (STZ)-induced type 1 diabetes mellitus (T1DM).^{23,24} In a separate study, NS-398-mediated COX-2 inhibition blunted glomerular hyperfiltration in STZ group of rats, which correlated with decreased glomerular prostaglandin production.²⁵ The latter study supports the notion that glomerular hemodynamic alterations early in DN involve the actions of COX-2-derived prostaglandins.²⁶ As for progressive renal disease, work by Wang et al^{27,28} found that cortical COX-2 expression increases in the rat remnant kidney model and that proteinuria and glomerulosclerosis can be attenuated using SC-58326, a selective COX-2 inhibitor. In addition, SC-58326 administration decreased markers of DN, including mesangial matrix expansion and proteinuria in diabetic, salt-sensitive, hypertensive rats.²⁹ Likewise, NS-398 reduced albuminuria, GFR, and kidney fibronectin expression in Akita mice with T1DM.³⁰ Taken together, these data suggest that COX-2-derived PGE₂ signaling through specific EP receptors promotes renal dysfunction in hypertension and/or diabetes.

Which of the four EP subtypes mediates the actions of PGE₂ in these disease contexts remains incompletely resolved. Our laboratory found that the actions of the podocyte EP4 receptor (EP4) are maladaptive because podocyte-specific EP4 deletion ameliorated, whereas overexpression of an EP4 mutant resistant to ligand-induced desensitization exacerbated, albuminuria and glomerulosclerosis after 5/6 nephrectomy in mice. In addition, adenoviral-mediated EP4 overexpression in cultured mouse podocytes led to an adhesion defect when challenged with mechanical stretch, an *in vitro* surrogate of intraglomerular forces exacerbated by hyperfiltration in DN.³¹ Conversely, inhibition of the Gq- and Gi-coupled EP1 and EP3 receptors has proven to be beneficial in preventing end-organ damage in severely hypertensive mice³² and AngII-mediated hypertension.³³ Interestingly, a role for EP1 in DN was suggested by Makino et al,³⁴ who treated rats with STZ-induced T1DM with an orally active EP1 antagonist, which resulted in improved albuminuria and decreased fibrotic glomerular damage, an effect attributed to decreased mesangial fibronectin and TGF- β production. However, possible non-glomerular effects of this compound cannot be fully discounted because renal EP1 expression has been described

in the vasculature, cortical collecting duct, and proximal tubule (PT) cells.^{35,36} Given that pharmacologic EP1 inhibition improves renal function and filtration barrier integrity in DN rats,³⁴ we hypothesized that gene-targeted EP1 deletion would attenuate DN-induced glomerular and/or tubular damage in diabetic mice. To this end, T1DM was induced in wild-type (WT) and EP1-null (*EPI*^{-/-}) mice on an FVB/n background using either low-dose STZ or genetic OVE26 models. Compared with their diabetic WT counterparts, diabetic *EPI*^{-/-} STZ or OVE26*EPI*^{-/-} mice were significantly less albuminuric and had decreased glomerular and tubular damage. Our data suggest that the PGE₂ EP1 receptor promotes glomerular and/or tubular dysfunction in diabetic mice, further implicating COX-derived PGE₂ in mediating deleterious consequences in diseases characterized by compromised renal hemodynamics.

Materials and Methods

Antibodies and Chemical Reagents

Polyclonal goat antimegalin (P-20), polyclonal rabbit anti-connective tissue growth factor (CTGF, H-55), anti-Wilm tumor 1 (WT1, C-19), and monoclonal anti- α -actin (1A4) were purchased from Santa Cruz Biotech (Santa Cruz, CA); polyclonal rabbit antifibronectin (F3648) was purchased from Sigma (St. Louis, MO). Secondary fluorescent Alexa Fluor 488 donkey anti-rabbit antibody was purchased from Molecular Probes (Burlington, Ontario). The EP1 antagonist AH6809 was purchased from Cayman Chemical (Ann Arbor, MI) and ONO-8711 (Sigma-Aldrich Canada, Oakville, ON, Canada).

Animals

Global EP1 knockout mice (*EPI*^{-/-}), generated and characterized by the Breyer group in 2007,³⁷ were used in this study after backcrossing for 10 generations onto the FVB/n background. Following guidelines established by the Diabetic Complications Consortium,³⁸ T1DM was induced in WT and *EPI*^{-/-} mice via the low-dose STZ model. Briefly, 8- to 10-week-old male mice were subjected to 5-day 50 mg/kg⁻¹ body weight (BW)⁻¹ intraperitoneal injections of STZ (Sigma) or 0.1 mol/L sodium citrate buffer (pH 4.5) as vehicle. Mice were followed up for 16 weeks after STZ until sacrifice. The transgenic OVE26 model of T1DM was also studied. Previously characterized³⁹ and commercially available OVE26 mice (The Jackson Laboratory, Bar Harbor, ME) on an FVB/n background were obtained at 4 weeks of age and intercrossed with *EPI*^{-/-} mice, yielding an OVE26*EPI*^{-/-} genotype. These groups were studied until up to 26 weeks of age. Experimental animals had free access to food and water. All operations were performed with the animals under anesthesia. Protocols were approved by the University of Ottawa Animal Care Committee and

conducted according to the guidelines of the Canadian Council on Animal Care.

Physiologic Data, Plasma Analysis, and Urinary PGE₂

At sacrifice, blood was collected via heparinized syringes kept on ice and centrifuged at $9000 \times g$ for 10 minutes at 4°C. Collected plasma was immediately frozen and kept at -80°C until subsequent analysis. For the STZ study, plasma glucose levels were determined by glucometry whereas the OVE26 study plasma samples were analyzed commercially (IDEXX Labs, Toronto, ON, Canada). Urine was collected in metabolic cages, and 24-hour volumes were recorded for each mouse. The urine was stored at -80°C. The PGE₂ urinary metabolite 13,14-dihydro-15-keto PGE₂ (PGEM), was assayed by a competitive enzyme immunoassay (Cayman Chemical) according to the manufacturer instructions. Briefly, urine samples and the PGEM standard were derivatized overnight at 37°C and assayed in triplicate using a 1:2 dilution of the original sample. Quantification was based on a colorimetric reaction catalyzed by acetylcholinesterase, after 90 minutes of incubation with Ellman's reagent. The plate was read at 420 nm, and PGEM was determined using the corresponding standard curve. All samples were expressed as picogram of PGEM per 24 hours.

Systolic BP Measurement and FITC-Inulin Clearance

Before sacrifice, systolic BP was measured via tail-cuff plethysmography (BP 2000; Visitech Systems, Apex, NC) in a subset of mice from each group. After a 5-day training regimen (10 BP readings per day), the mean daily systolic BP was calculated from 5 consecutive days of measurements (5 preliminary and 10 actual BP readings per day). In parallel, fluorescein isothiocyanate (FITC)-labeled inulin clearance was used to estimate GFR. Briefly, 5% (w/v) FITC-inulin (Sigma) dissolved in 0.9% (w/v) saline was dialyzed overnight and sterilized by filtration. Anesthetized mice received a 3.74-μL/g bolus of FITC-inulin retro-orbitally. Approximately 20-μL blood samples were collected from the saphenous vein into heparinized capillary tubes and centrifuged for 10 minutes at 10,000 rpm. Blood sampling was performed at 3, 7, 10, 15, 35, 55, and 75 minutes after injection. Samples were buffered in 500 mmol/L HEPES (pH 7.4), and plasma fluorescence was measured (Excitation 488 nm/Emission 538 nm). A two-compartment clearance model was used to calculate GFR as previously described⁴⁰ using statistical analysis software (Graphpad Prism version 5.02; GraphPad, San Diego, CA).

Albuminuria

At selected time points, nondiabetic and diabetic mice were subjected to 24-hour urine collection in metabolic cages for subsequent urinalysis. At the 8-week time point, mice were acclimatized to the metabolic cages for 4 hours on the morning of collection. Mice had free access to drinking water and chow.

After collection, samples were kept on ice, samples were centrifuged at 3000 rpm for 10 minutes to pellet urinary sediment, and aliquots stored at -80°C until analysis. Albuminuria was measured using the Mouse Albumin ELISA Kit (Bethyl Labs, Montgomery, TX) following the manufacturer protocol. Extrapolated albumin concentrations were normalized to 24-hour urine volume and creatinine concentrations as determined by the Creatinine Companion Kit (Exocell, Philadelphia, PA).

PAS Scoring and Immunostaining of Kidney Sections

At sacrifice, mice were anesthetized and perfused with PBS, and kidneys were excised, dissected, and immediately fixed in 4% paraformaldehyde. Paraffin-embedded kidney sections (3 μm) were obtained and stained with periodic acid-Schiff (PAS) reagent. All sectioning, paraffin embedding, and PAS staining were performed by the University of Ottawa's pathology department. PAS-stained kidney sections were viewed under a light microscope at $\times 400$ magnification (Axioskop 2 Imager A1; Carl Zeiss, Jena, Germany). Representative glomerular cross-sectional profiles for each group were analyzed in a masked manner. Imaging software (Axiovision version 4.8; Carl Zeiss) was used to calculate relative mesangial matrix and glomerular area.

Fibronectin, megalin, and α -actin immunohistochemistry (IHC) was performed on paraffin-embedded sections mounted on glass slides. Sections were deparaffinized in mixed xylenes (Thermo-Fisher Scientific, Ottawa, ON, Canada) and rehydrated through a gradient of ethanols and distilled water. Sections were washed three times in PBS and boiled for 20 minutes in 0.1 mol/L sodium citrate buffer (pH 6.0) for antigen unmasking, and endogenous peroxidase activity was quenched by 0.3% H₂O₂ in methanol. Sections were blocked in 5% rabbit or goat serum (Vector Laboratories, Burlingame, CA) for 1 hour and incubated with antifibronectin (1:200), antimegalina (1:100), or anti- α -actin (1:200) overnight at 4°C. Slides were then incubated with horseradish peroxidase- or FITC-labeled rabbit or goat secondary antibodies, respectively. Sections were then processed for Vector ABC/3,3'-diaminobenzidine staining according to the manufacturer instructions (Vector Laboratories). Diaminobenzidine exposure times were identical for all samples. Slides were then dehydrated and covered with mounting media (Vector Laboratories) and coverslips. Slides were visualized under light or fluorescence microscope whereby representative cortical profiles from each group were obtained in a masked manner. Positive signal area was calculated using the AlphaView software suite version 3.0.3 (Alpha Innotech, Santa Clara, CA).

Immunofluorescence detection of podocyte WT1 was used to estimate podocyte numbers. Frozen optimal cutting temperature embedded kidneys were sectioned (8 μm) and processed for WT1 staining. After brief acetone fixation, slides were dried at room temperature, washed in PBS, and blocked with 5% donkey serum (Jackson Laboratory), followed by incubation with a primary anti-WT1 antibody diluted at 1:200 overnight. After several washes, a

fluorescently conjugated secondary donkey anti-rabbit antibody (1:1000) was added for 1 hour at room temperature. Sections were covered with fluorescent mounting medium (Vector Laboratories) and coverslips. Podocyte quantification and analysis were performed in a masked manner by fluorescence microscopy (Carl Zeiss).

RNA Extraction and qPCR

Snap frozen kidney cortex was mechanically homogenized using the TP-103 Amalgamator COEcapmixer (GC America Inc, Alsip, IL). Capsules and ceramic beads were dipped into liquid nitrogen before sample addition. Cells were homogenized using QIAshredder columns (Qiagen, Toronto, ON, Canada). RNA was extracted using the Qiagen RNEasy minikit per the manufacturer instructions. Extracted RNA was converted to cDNA using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) with 500 ng of starting material per reaction. Quantitative real-time PCR (qPCR) was performed using an ABI Prism 7000 Sequence Detection System with SYBR Advantage qPCR Premix (Clontech Laboratories, Mountain View, CA) according to the manufacturer instructions. Primers used were as follows: nephrin sense, 5'-CCCAACACTGGAAGAGGTGT-3' and antisense, 5'-CTGGTCGTAGATCCCCCTTG-3'; and megalin sense, 5'-AGGCCACCAGTTCCTTGTCT-3' and antisense, 5'-AGGACACGCCATTCTCTTG-3'.

Western Blotting

At sacrifice, kidney cortex was immediately snap frozen in liquid nitrogen and kept at -80°C . Tissue was homogenized by rotor-stator in tissue lysis buffer [150 mmol/L NaCl, 1% Triton X-100, and 50 mmol/L Tris (pH 8.0), 1% protease inhibitor cocktail (Sigma)] followed by brief sonication on ice. Samples were centrifuged at 13,000 rpm for 10 minutes at 4°C . Protein concentration was determined by the Bradford method (Bio-Rad Laboratories, Hercules, CA), and equal amounts were boiled for 5 minutes and resolved by 8% SDS-PAGE for fibronectin and CTGF and by commercially available 4% to 12% gradient gels (Bio-Rad Laboratories) for megalin. For fibronectin and CTGF, SDS-PAGE gels were transferred to nitrocellulose membranes, which were blocked in 5% milk, probed with primary antibody overnight, and stored in horseradish peroxidase-conjugated secondary antibody (Jackson Laboratory) for 1 hour at room temperature. For megalin, gels were transferred overnight (25 volts for 18 hours) at 4°C , and immunoblotting was performed as described above. Detection was effected by enhanced chemiluminescence (GE Healthcare, Buckinghamshire, United Kingdom) and densitometry performed using the Alpha Imager System (Alpha Innotech).

Previously characterized mouse PT (MCT) cells [provided by Dr. Eric Neilson (Vanderbilt University, Nashville, TN)]⁴¹ were grown to confluence; incubated with 1 $\mu\text{mol/L}$ PGE₂ and/or 100 nmol/L ONO8711; and lysed in RIPA

containing 0.5 mmol/L phenylmethylsulfonyl fluoride, 1% protease inhibitor cocktail, 1 mmol/L sodium pyrophosphate, 10 mmol/L sodium fluoride, and 100 $\mu\text{mol/L}$ sodium orthovanadate; and briefly sonicated. Protein was quantified with Bradford reagent (Bio-Rad). Samples were denatured at 70°C for 15 minutes, separated by electrophoresis, and transferred onto a nitrocellulose membrane. Membranes were blocked in 5% milk for 90 minutes and incubated overnight with 1:5000 antifibronectin followed by 1:2000 anti-rabbit antibody (Promega, Madison, WI) for 90 minutes. SuperSignal West Pico chemiluminescent reagents (Thermo-Fisher Scientific) was applied, and β -actin was detected as a loading control for densitometry.

Lucigenin Assay for Superoxide Production

Conditionally immortalized human podocytes⁴² were grown at 33°C on type I collagen (BD Biosciences, Franklin Lakes, NJ)-coated plastic culture dishes in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 U/mL of penicillin/streptomycin, and 10 U/mL of recombinant interferon- γ . Differentiation was induced by maintaining the cells at 37°C in the above media without recombinant interferon for 10 to 14 days. Cells were maintained in RPMI 1640 medium supplemented with 2% fetal bovine serum and 100 U/mL of penicillin/streptomycin for 2 days before treatment with the EP1 antagonist AH6809 for 1 hour with subsequent stimulation with 500 nmol/L AngII (EMD Millipore, Billerica, MA) for 2 hours to induce superoxide

Figure 1 Twenty-four hour urinary albumin excretion in STZ and OVE26 models of STZ-induced T1DM. Albumin concentration was measured by ELISA and normalized to 24-hour urine volume. **A:** Eight weeks after STZ administration. **B:** Sixteen weeks after STZ administration. **C:** OVE26 study at 8 weeks of age. **D:** OVE26 study at 26 weeks of age. Data are presented as means $\pm$ SEM of duplicate samples (STZ study: WT, $n = 8$; WTSTZ, $n = 9$; EP1^{-/-}, $n = 6$; EP1^{-/-}STZ, $n = 11$; OVE26 study: 8 weeks: WT, $n = 5$; OVE26, $n = 14$; EP1^{-/-}, $n = 8$; OVE26EP1^{-/-}, $n = 9$; 26 weeks: WT, $n = 6$; OVE26, $n = 6$; EP1^{-/-}, $n = 8$; OVE26EP1^{-/-}, $n = 7$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Table 1 Physiologic Parameters of the STZ Study

Parameter	WT	WTSTZ	EP1 ^{-/-}	EP1 ^{-/-} STZ
BW (g)	30.2 ± 0.5	25.8 ± 0.7*	29.4 ± 0.7	24.5 ± 0.5*
Kidneys/BW (mg/g)	12.6 ± 0.2	20.5 ± 0.9*	14.2 ± 0.2	21.1 ± 0.6*
Plasma glucose (mmol/L)	6.8 ± 0.5	30.6 ± 1.4*	7.7 ± 0.2	31.5 ± 0.4*

**P* < 0.01 versus healthy controls.

BW, body weight.

production. Cells were harvested in ice cold phosphate buffer (50 mmol/L KH₂PO₄, 1 mmol/L EGTA, and 150 mmol/L sucrose) (pH 7.4) with protease inhibitors. Fifty microliters of cell lysate was added to 175 μ L of buffer and 1.25 μ L of 1 mmol/L lucigenin (ENZO Life Sciences, Farmingdale, NY). Baseline activity was measured. Cells were stimulated by addition of 25 μ L of 1 mmol/L NADPH, and active levels were measured. Baseline activity reported as relative light units (RLU) was subtracted, and the adjusted RLU value was normalized to protein concentration.

Myography

Wire myography was used to assess microvascular contractility in response to AngII in experimental mice. Briefly, second-order branches of mesenteric arteries were removed from anesthetized mice, placed in Krebs solution, and cleaned of connective tissue. Arteries were mounted in a Multi Wire Myograph System (DMT, Ann Arbor, MI). Maximal vessel contractility was assessed on addition of 60 mmol/L KCl plus 10 μ mol/L norepinephrine. Arteries were then washed and challenged with 10 nmol/L AngII. The AngII-induced contraction response was calculated as a percentage of maximal constriction.

Electron Microscopy

Ultrastructural analysis of the glomerular filtration barrier was assessed by transmission electron microscopy (TEM). Kidneys were immersion fixed in cold 2.4% glutaraldehyde in PBS buffer, postfixed in 2% buffered osmium tetroxide, dehydrated in graded ethanols, and embedded in Spurr resin. Samples were sectioned at 70 nm, placed on copper for TEM, and stained with uranyl acetate and lead citrate. Samples were screened on a Hitachi H-7100 microscope (Hitachi, Tokyo, Japan). Representative profiles at $\times 5000$ and $\times 20,000$ magnifications from two to three glomeruli were assessed in three mice per group. Glomerular basement membrane (GBM) measurements were taken in random capillary loops, while avoiding proximity to mesangial cells.

Statistical Analysis

The values are presented as means $\pm$ SEM. Statistical comparisons between two-groups was performed using the unpaired Student's *t*-test, whereas analysis of variance (analysis of variance) was used for three or more groups, followed by a Newman-Keuls posttest. Statistical significance was achieved when *P* < 0.05.

Results

EP1 Deletion Ameliorates Albuminuria in Two Distinct Models of T1DM

Pharmacologic inhibition of the PGE₂ EP1 receptor using an orally active antagonist decreases albuminuria in rats with STZ-induced T1DM.³⁴ Therefore, we hypothesized that mice with global EP1 deletion would likewise be protected. As shown in Figure 1, A and B, both the WTSTZ and EP1^{-/-}STZ groups had similar, albeit elevated, urinary albumin levels compared with healthy controls at the 8-week time point. However, at 16 weeks, WTSTZ mice developed more severe albuminuria, whereas EP1^{-/-}STZ values did not increase to a similar degree, as measured by 24-hour urinary albumin excretion rates (1546 $\pm$ 282 for WTSTZ mice versus 525 $\pm$ 110 μ g/24 hours for EP1^{-/-}STZ mice, *P* < 0.001). Both STZ groups developed T1DM phenotypes characterized by slight weight loss, kidney hypertrophy, and polyuria (Table 1). Plasma glucose levels were elevated similarly in WT and EP1^{-/-}STZ animals, which is consistent with equivalent diabetes induction in both groups.

Next, we extended our findings to the more robust OVE26 transgenic model of DN.^{39,43} OVE26EP1^{-/-} mice were protected from albuminuria as early as 8 weeks of age (Figure 1C). OVE26 mice continued to be significantly more albuminuric than OVE26EP1^{-/-} mice at 26 weeks of age (2762 $\pm$ 767 in the OVE26 mice versus 1022 $\pm$ 395 μ g/24 hours in the OVE26EP1^{-/-} mice, *P* < 0.05). At 26 weeks of age, kidney hypertrophy was exacerbated in OVE26 mice compared with OVE26EP1^{-/-} mice, whereas

Table 2 Physiologic Parameters of the OVE26 Study

Parameter	WT	OVE26	EP1 ^{-/-}	OVE26EP1 ^{-/-}
BW (g)	27.1 ± 0.9	22.8 ± 1.1*	26.6 ± 1.1	21.2 ± 0.9*
Kidneys/BW (mg/g)	14.5 ± 0.6	21.7 ± 0.6**	13.5 ± 0.2	18.9 ± 1.5**†
Plasma glucose (mmol/L)	9.5 ± 0.8	38.3 ± 3.1**	10.6 ± 0.6	38.6 ± 2.0**

P* < 0.05, *P* < 0.001 versus healthy controls.

†*P* < 0.01 versus OVE26.

Figure 2 Urine PGE₂ levels in OVE26 mice at 26 weeks. Samples were obtained by 24 hours via metabolic cage collection and subjected to PGEM enzyme immunoassay. Values were normalized to 24-hour urine volume. Statistical data are presented as means $\pm$ SEM ($n = 5$ mice per group). ** $P < 0.01$.

plasma biochemistry confirmed that the protective effect was independent of the diabetes-associated hyperglycemia, which reached similar degrees in both groups (Table 2). The observed reduction in overall urinary albumin excretion in the OVE26EP1^{-/-} cohort was not a consequence of altered renal PGE₂ production because urinary levels of PGEM were similarly increased in both diabetic groups (Figure 2). Deletion of the *PTGER1* gene did not alter mRNA expression of the other EP receptor subtypes (data not shown).

Decreased Albuminuria in EP1^{-/-}STZ Mice Occurs Independently of GFR and BP

To determine whether the increase in albuminuria observed in the WTSTZ mice compared with the EP1^{-/-} mice after 4 months of diabetes was due to differences in glomerular hyperfiltration, we estimated GFR based on FITC-inulin clearance. As expected, hyperfiltration was evident in both the WTSTZ and EP1^{-/-}STZ cohorts, with 2.5-fold increases

in GFR ($10.3 \pm 2.0 \mu\text{L}/\text{min}^{-1}/\text{g BW}^{-1}$ for the WT cohort versus $26.1 \pm 3.0 \mu\text{L}/\text{min}^{-1}/\text{g BW}^{-1}$ for the WTSTZ cohort and $11.1 \pm 2.5 \mu\text{L}/\text{min}^{-1}/\text{g BW}^{-1}$ for the EP1^{-/-} cohort versus $28.6 \pm 3.8 \mu\text{L}/\text{min}^{-1}/\text{g BW}^{-1}$ EP1^{-/-}STZ, $P < 0.05$) at 16 weeks after STZ administration (Figure 3A). Furthermore, no significant changes in tail-cuff systolic BP were noted between any of the STZ groups (Figure 3B).

OVE26EP1^{-/-} Mice Hyperfilter to a Lesser Extent Than OVE26 at 26 Weeks Independently of BP

FITC-inulin clearance was also used to estimate GFR in OVE26 mice at 26 weeks of age. As shown in Figure 3C, a 2.5-fold increase in FITC-inulin clearance was observed in OVE26 mice, indicative of glomerular hyperfiltration;

Figure 3 FITC-inulin clearance and systolic BP measurement. **A** and **C**: Before sacrifice, mice were subjected to FITC-inulin injections, and plasma FITC fluorescence was measured at 488 nm. Data are presented as means $\pm$ SEM (STZ study: $n = 5$ mice per group; OVE26 study: WT, $n = 5$ and OVE26, $n = 13$; EP1^{-/-}, $n = 9$; OVE26EP1^{-/-}, $n = 5$). **B** and **D**: Systolic BP was measured by tail-cuff plethysmography. Data are presented as means $\pm$ SEM of 5 days of independent measurements for 10 readings per day (STZ study: WT, $n = 8$; WTSTZ, $n = 8$; EP1^{-/-}, $n = 9$; EP1^{-/-}STZ, $n = 8$; OVE26 study: WT, $n = 11$; OVE26, $n = 8$; EP1^{-/-}, $n = 7$; OVE26EP1^{-/-}, $n = 6$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Figure 4 PAS staining in both studies. Representative images of paraformaldehyde-fixed, paraffin-embedded kidney sections stained with PAS at 16 weeks for the STZ study (**A**) and 26 weeks for the OVE26 study (**B**). Original magnification, $\times 400$.

Figure 5 Glomerular mesangial expansion and hypertrophy measurements in STZ and OVE26 models of STZ-induced T1DM. Glomerular surface area (A and C) and mesangial matrix scoring (B and D) in the STZ and OVE26 studies at 16 and 26 weeks, respectively. Statistical data are presented as means $\pm$ SEM ($n = 5$ to 7 mice per group, 25 fields per mouse). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

however, diabetic *EPI*^{-/-} mice exhibited milder hyperfiltration versus OVE26 mice (WT, $10.2 \pm 2.1 \mu\text{L}/\text{min}^{-1}/\text{g BW}^{-1}$; OVE26, $23.7 \pm 1.4 \mu\text{L}/\text{min}^{-1}/\text{g BW}^{-1}$; *EPI*^{-/-}, $10.9 \pm 1.3 \mu\text{L}/\text{min}^{-1}/\text{g BW}^{-1}$; and OVE26*EPI*^{-/-}, $15.9 \pm 1.7 \mu\text{L}/\text{min}^{-1}/\text{g BW}^{-1}$, $P < 0.05$). Diabetes had no effect on systolic BP in each of the OVE26 groups (Figure 3D).

EP1 Deletion Reduces the Extent of Mesangial Matrix Expansion and Glomerular Hypertrophy in the STZ and OVE26 Models

Structural analysis of PAS-stained paraffin-embedded kidney sections was performed to determine whether the protective effect of genetic EP1 deletion on DN-induced albuminuria was associated with decreased glomerular damage. As shown in Figures 4 and 5, both the STZ and OVE26 models presented with increased mesangial matrix deposition, which was attenuated in *EPI*^{-/-} mice (percentage of glomerular area: STZ study: WT, $22\% \pm 1\%$; WTSTZ, $36\% \pm 6\%$; *EPI*^{-/-}, $22\% \pm 1\%$; *EPI*^{-/-}STZ, $26\% \pm 4\%$; and OVE26 study: WT, $22\% \pm 4\%$; OVE26, $35\% \pm 8\%$; *EPI*^{-/-}, $23\% \pm 6\%$; OVE26*EPI*^{-/-}, $26\% \pm 8\%$). Similarly, although glomerular hypertrophy was elevated in both diabetic models, it was lower in the *EPI*^{-/-} groups (glomerular area: STZ study: WT, $6 \pm 1 \text{ mm}^2$; WTSTZ, $10 \pm 3 \text{ mm}^2$; *EPI*^{-/-}, $8 \pm 2 \text{ mm}^2$; *EPI*^{-/-}STZ, $8 \pm 3 \text{ mm}^2$; and OVE26 study: WT, $7 \pm 1 \text{ mm}^2$; OVE26, $11 \pm 3 \text{ mm}^2$; *EPI*^{-/-}, $7 \pm 2 \text{ mm}^2$; OVE26*EPI*^{-/-}, $8 \pm 3 \text{ mm}^2$).

WT1-Positive Cell Counts Are Similar in Diabetic WT and *EPI*^{-/-} Glomeruli

Podocyte depletion, caused by either apoptosis or detachment, is commonly observed in DN.^{44–46} To further evaluate the extent of glomerular filtration barrier damage, we

counted podocyte numbers in both the STZ and OVE26 mouse cohorts. As depicted in Figure 6, diabetic *EPI*^{-/-} mice had a similar reduction in WT-1–positive nuclei compared with WT mice in both diabetic models (WT1-positive cells per glomerulus: STZ study: WT, 15.2 ± 0.6 ; WTSTZ, 11.5 ± 0.8 ; *EPI*^{-/-}, 15.9 ± 0.8 ; *EPI*^{-/-}STZ, 11.8 ± 0.6 ; OVE26 study: WT, 17.1 ± 0.5 , OVE26, 14.2 ± 0.3 ; *EPI*^{-/-}, 18.2 ± 0.6 ; OVE26*EPI*^{-/-}, 13.6 ± 0.4 , $P < 0.05$).

DN-Induced GBM Thickening and Foot Process Derangement Are Reduced in OVE26*EPI*^{-/-} Mice

TEM was used to assess glomerular filtration barrier integrity in a subset of mice from the OVE26 study. Increased albumin leakage and glomerular structural damage seen in these diabetic mice was associated with foot process effacement (Figure 7A) and augmented GBM width (Figure 7B), which were not apparent in the OVE26*EPI*^{-/-} cohort (WT, $159 \pm 3 \text{ nm}$; OVE26, $214 \pm 36 \text{ nm}$; *EPI*^{-/-}, $160 \pm 2 \text{ nm}$; OVE26*EPI*^{-/-}, $175 \pm 10 \text{ nm}$; $P < 0.05$).

EP1 Antagonism Reduces DN-Induced Nephron mRNA Down-Regulation in Diabetic Mice and AngII-Induced Podocyte-Derived Superoxide Production in Culture

Because deletion of the EP1 receptor was associated with glomerular filtration barrier protection, we hypothesized that EP1 targeting may have a beneficial effect on the podocyte slit diaphragm in DN. To this end, we measured nephrin mRNA levels in the cortex of the OVE26 study mice. Renal nephrin mRNA levels were significantly reduced in OVE26 mice but were preserved in OVE26*EPI*^{-/-} mice (Figure 8A). Higher nephrin levels may be indicative of preserved

Figure 6 Glomerular podocyte estimation in STZ and OVE26 models. Mice were sacrificed and kidneys processed for frozen sectioning and WT1 antibody immunofluorescence microscopy. A: Representative glomeruli from healthy and diabetic mice. B: Representation of the STZ study. C: OVE26 study WT1-positive podocytes per glomeruli. A total of 15 to 20 glomeruli per mouse were assessed in each group. Statistical data are presented as means $\pm$ SEM ($n = 4$ to 6 mice per group). * $P < 0.05$. Original magnification, $\times 400$ (A).

Figure 7 TEM in the OVE26 study. Glutaraldehyde-fixed cortex samples were processed for TEM. **A:** Representative micrographs from each group. **B:** GBM thickness measurements. A total of 20 to 30 representative micrographs from two to three glomeruli per mouse, $n = 3$ mice per group. Statistical data are presented as means $\pm$ SEM. * $P < 0.05$.

function in the remaining podocytes, which is consistent with improved filtration barrier integrity in diabetic *EPI*^{-/-} mice.

Recent studies have found that AT1 receptor activation is partly dependent on EP1 and/or EP3 receptor signaling.³³ Although the specific interaction between these two receptor families remains incompletely described, they often have synergistic effects, including reactive oxygen species (ROS) generation and/or increasing vascular dysfunction.^{47–49} Because increased oxidative stress due to higher levels of ROS can result in podocyte damage and ultimately the development of albuminuria,⁵⁰ we determined whether EP1 ablation would reduce AngII-mediated ROS generation. As shown in Figure 8B, AngII-induced superoxide production in conditionally immortalized human podocytes, as assessed by the lucigenin assay, indicated that antagonism of the EP1 receptor with AH6809 abrogated the AngII-mediated superoxide production.

EPI^{-/-} Diabetic Mice Have Reduced Expression of Renal Fibrosis Markers

In addition to filtration barrier damage, diabetes promotes tubular dysfunction, leading to interstitial fibrosis. We

therefore measured cortical fibronectin expression as an indication of kidney fibrotic damage. Immunoblotting of renal lysates revealed that fibronectin expression was up-regulated in WTSTZ mice but was unchanged in *EPI*^{-/-}STZ mice compared with healthy controls (Figure 9) (809 ± 82 AU for WT mice versus 2060 ± 212 AU for WTSTZ mice and 1335 ± 232 AU for *EPI*^{-/-} mice versus 1327 ± 75 AU for *EPI*^{-/-}STZ mice, $P < 0.01$). In parallel, fibronectin expression as detected by IHC revealed elevated staining in WTSTZ mice with a trend toward reduction noted in the *EPI*^{-/-}STZ mice compared with healthy controls. However, these data did not reach statistical significance.

In the OVE26 study, markers of DN-induced renal damage that were assessed included fibronectin, CTGF, and α -actin. The OVE26 phenotype was associated with an increase in renal fibronectin (Figure 10) expression; however, immunoblotting revealed that *EPI*^{-/-} mice had decreased overall fibronectin levels (WT, 2957 ± 421 AU; OVE26, 6247 ± 444 AU; *EPI*^{-/-}, 1790 ± 212 AU; OVE26EPI^{-/-}, 2895 ± 299 AU; $P < 0.01$). Because the OVE26 model typically induces a more robust diabetic phenotype compared with STZ, we measured renal CTGF expression. The CTGF protein expression was decreased in OVE26EPI^{-/-} mice compared with OVE26 mice (WT,

Figure 8 Nephlin qPCR in the renal cortex and ROS generation in human podocytes. **A:** Kidney cortex RNA was reverse transcribed and qPCR was performed using SYBR Green. Data are reported using the $\Delta\Delta CT$ method and expressed as fold WT normalized to GAPDH. Statistical analysis is presented as means $\pm$ SEM from four to six mice per group assayed in triplicate. * $P < 0.05$. **B:** Cultured human podocytes were differentiated for 14 days and subjected to 2 hours. Five-hundred-nanomolar AngII stimulations with or without pretreatment with the EP1 receptor antagonist AH6809 are shown. Data are presented as mean RLU/μg of protein $\pm$ SEM from four experiments. * $P < 0.05$.

Figure 9 Renal fibronectin expression in STZ mice. Paraffin-embedded kidney sections were processed for IHC with antifibronectin antibody and visualized by light microscopy. **A:** Representative image of fibronectin IHC in the cortex. **B:** Analysis of immunodetectable fibronectin expression. **C:** Representative fibronectin and β -actin Western blots in mouse kidney cortex. Ten micrograms of protein resolved by 8% SDS-PAGE Tris-HCl gel. **D:** Fibronectin Western blot as determined by densitometric analysis. Statistical analysis is presented as means $\pm$ SEM in three to five mice per group. * $P < 0.05$, ** $P < 0.01$. Original magnification: $\times 200$ (A).

1987 $\pm$ 150 AU; OVE26, 3528 $\pm$ 313 AU; EP1^{-/-}, 2105 $\pm$ 271 AU; OVE26EP1^{-/-}, 2212 $\pm$ 313 AU; $P < 0.05$). Moreover, α -actin staining was assessed as an additional marker of interstitial fibrosis. As shown in Figure 11, basal

α -actin staining was observed in vascular structures in all mice; however, the presence of α -actin-positive cells was markedly elevated in the interstitium of OVE26 mice, an effect observed to a lesser extent in the EP1^{-/-} cohort.

EP1 Receptor Mediates PGE₂-Induced Fibronectin Expression in Cultured MCT Cells

To further explore the protective effect of EP1 deletion on diabetes-induced cortical fibronectin up-regulation, we tested whether PGE₂ stimulates fibronectin expression in cultured MCT cells. To this end, MCT cells were stimulated with either PGE₂ alone or in combination with the EP1 receptor antagonist ONO8711. PGE₂ stimulated fibronectin expression by two-fold at 24 hours, which was abrogated entirely by ONO8711 (Figure 12).

EP1^{-/-}STZ and OVE26EP1^{-/-} Mice Have Preserved Tubular Megalin Expression

Tubular damage in diabetes is often associated with increased glucose and sodium reabsorption, cellular hypertrophy, and impaired albumin reabsorption.^{51,52} Because megalin participates in postglomerular albumin processing along the PT, we tested whether the reduced albuminuria seen in diabetic EP1^{-/-} mice was accompanied by attenuated tubular damage, as measured by megalin expression. Immunodetectable megalin protein was significantly decreased in OVE26 and STZ mice (Figure 13), whereas diabetic EP1^{-/-} mice were protected against megalin loss (STZ study: WT, 1135 $\pm$ 89 AU; WTSTZ, 791 $\pm$ 48 AU; EP1^{-/-}, 1062 $\pm$ 63 AU; EP1^{-/-}STZ, 1091 $\pm$ 124 AU; OVE26 study: WT, 993 $\pm$ 148 AU; OVE26, 630 $\pm$ 101 AU; EP1^{-/-}, 940 $\pm$ 53 AU; OVE26EP1^{-/-}, 1094 $\pm$ 185 AU). Similar findings were observed by immunoblotting for megalin protein in the STZ study cortex samples

Figure 10 Renal fibronectin and CTGF immunoblotting in OVE26 mice. **A:** Representative Western immunoblot of fibronectin and CTGF protein expression in mouse kidney cortex. Fifteen micrograms of protein resolved by 8% Tris-HCl SDS-PAGE. Representation of fibronectin (B) and CTGF (C) Western blot as determined by densitometric analysis. Statistical analysis is presented as means $\pm$ SEM in four to six mice per group. * $P < 0.05$, ** $P < 0.01$.

Figure 11 α -Actin staining in the OVE26 study. Paraffin-embedded kidney sections were processed for immunofluorescence staining with anti- α -actin antibody and visualized by fluorescence microscopy. **A:** Representative images of α -actin in the cortex. **B:** Analysis of immunodetectable α -actin expression. Statistical analysis is presented as means $\pm$ SEM in three to five mice per group. * $P < 0.05$, ** $P < 0.01$. Original magnification: $\times 200$ (**A**).

(Figure 13E), whereas mRNA levels were decreased in both cohorts (Figure 13F).

OVE26EP1^{-/-} Mice Are Less Sensitive to AngII-Induced Mesenteric Artery Vasoconstriction

In addition to expression in glomerular and tubular cells, the EP1 receptor is also found in vascular smooth muscle cells along with the angiotensin AT1 receptor, where it likely contributes to vasoconstriction. To assess whether loss of EP1 receptor expression would affect AngII-mediated vasoconstriction, we isolated mesenteric arteries from a subset of the OVE26 study mice at 30 weeks of age or diabetes and subjected them to wire myography. As shown in Figure 14, mesenteric arteries isolated from OVE26 diabetic mice exhibited a significantly enhanced AngII-induced vasoconstriction. However, both the maximal AngII-induced contraction and the rate of vasoconstriction were markedly reduced in vessels obtained from OVE26EP1^{-/-} mice.

Discussion

COX inhibitors (eg, nonsteroidal anti-inflammatory drugs) that block the synthesis of prostaglandins, thromboxanes,

and prostacyclins (ie, the prostanoids) are reported to reduce DN-associated proteinuria.^{53,54} Although COX inhibition is antiproteinuric, nonsteroidal anti-inflammatory drugs and the recently developed gastrointestinal-sparing COX-2 selective inhibitors (coxibs) can be nephrotoxic for renal disease patients.^{55,56} By blocking the synthesis of vasodilatory prostaglandins, these drugs can elicit a precipitous decrease in renal blood flow and GFR.^{16,57–60} Moreover, some of the clinical data with COX-2 inhibitors have failed to demonstrate beneficial antiproteinuric effects.⁶¹ Such discrepant outcomes are likely because nonsteroidal anti-inflammatory drugs block the synthesis of an entire family of COX-derived prostanoids, which exert numerous biological actions through a host of cell surface receptors. More effective strategies might therefore focus downstream of COX blockade and differentiate between those prostanoids and their respective receptors that deliver protective effects from those that impair renal function.

We studied the role of the EP1 receptor on the progression of DN in mice. Using two distinct models of T1DM, we observed a 60% reduction in urinary albumin excretion and decreased renal structural and ultrastructural damage in mice with global EP1 deletion, suggestive of partial yet significant preservation of glomerular filtration barrier integrity. Furthermore, our data indicate that the PGE₂ EP1 receptor promotes renal and glomerular hypertrophy, mesangial matrix expansion, and indications of tubulointerstitial fibrosis. The limited renal damage in the EP1^{-/-} cohorts was independent of the diabetic status of the mice because all groups displayed similar hyperglycemia. Our results using a gene-targeted approach are consistent with previously published data that found beneficial effects of pharmacologic antagonism of the EP1 with ONO8713 on albuminuria and mesangial cell dysfunction in rats with STZ-induced diabetes.³⁴ To our knowledge, our study is the first to identify

Figure 12 MCT cell fibronectin expression. MCT cells were grown to confluence and stimulated with 1 μ mol/L PGE₂, 100 nmol/L ONO8711, or both for 24 hours and samples subjected to Western immunoblotting. Densitometric analysis (**A**) and representation (**B**) of fibronectin and β -actin are shown. Statistical analysis is presented as means $\pm$ SEM from four to six experiments. * $P < 0.05$.

Figure 13 Kidney megalin expression in STZ and OVE26 models of STZ-induced T1DM. **A** and **C**: Representative images of immunodetectable megalin protein by IHC. **B** and **D**: Representation of IHC scoring. **E**: Megalin immunoblotting. Fifteen micrograms of kidney cortex protein was resolved in 4% to 12% gradient Tris-HCl gel ($n = 4$ to 6 per group) and probed with a megalin antibody. **F**: Megalin qPCR. Kidney cortex mRNA was isolated and megalin qPCR was performed in the OVE26 study using SYBR Green on cDNA ($n = 5$ per group). Statistical analysis is presented as means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$.

EP1 receptor actions at the PT because immunodetectable levels of megalin were significantly preserved in diabetic $EP1^{-/-}$ mice and EP1 antagonism reduced PGE₂-mediated fibronectin up-regulation in a PT cell line. Furthermore, recent data implicate EP1 in promoting end-organ damage in severely hypertensive mice because of increased susceptibility to developing aortic aneurysms.³² Abolishing EP1 expression in our diabetic mice had no effect on BP because it remained unchanged with the onset of diabetes. However, a striking disparity was noted regarding mouse survival because OVE26 $EP1^{-/-}$ mice fared better than age-matched diabetic controls (data not shown).

The *PTGER1* gene encodes a seven-transmembrane receptor that uses the G α_q (Gq) signaling axis whereby PGE₂ binding to the EP1 leads to the activation phospholipase C β , which catalyzes phosphoinositide hydrolysis, calcium mobilization, and protein kinase C activation.¹⁶ Renal EP1 expression has been described in mesangial cells, podocytes, collecting duct cells, the vasculature, and PT cells.^{15,34,35} Makino et al³⁴ attributed the beneficial effect

seen by EP1 antagonism with ONO8713 in DN rats to decreased mesangial cell fibronectin and TGF- β production at the transcriptional level. Other studies found that the hypertrophic response of cultured rat mesangial cells to AngII could be blocked by pharmacologic EP1 antagonism.⁴⁸ Our findings are consistent with such observations as glomerular hypertrophy and mesangial matrix expansion that was significantly blunted in diabetic $EP1^{-/-}$ mice. Although the impact of altered mesangial cell homeostasis is important because these cells help support glomerular architecture, the extent of podocyte damage or loss was not investigated in those studies.

Podocytes maintain filtration barrier integrity by establishing the size and charge selective 40-nm-wide slit diaphragm.⁶² Increased Gq signaling can be detrimental to podocyte health because transgenic expression of a constitutively active Gq subunit in podocytes of mice induced COX-2 via calcineurin/nuclear factor of activated T cells activation and promoted podocyte apoptosis.^{63,64} A role for COX-2 in podocyte injury has also been proposed, whereby podocyte-specific overexpression of COX-2 in mice increases doxorubicin-induced albuminuria and foot process effacement, an effect that can be blocked using the COX-2 selective inhibitor NS398.⁶⁵ Because Gq activation predisposes podocytes to damage via COX-2 induction, the renoprotective effect of EP1 deletion may be in part due to decreased signaling of this receptor subtype in podocytes. In our study, we observed minimal podocytopenia in diabetic mice with no significant differences noted between WT and $EP1^{-/-}$ mice. Yet, in culture, EP1 antagonism had an inhibitory effect on AngII-mediated podocyte superoxide

Figure 14 Myography on isolated mesenteric arteries from the OVE26 study. Mesenteric arteries were removed from anesthetized mice, placed in Krebs solution, and mounted in a wire Multi Myograph System. Maximal contraction was achieved by stimulation with 60 mmol/L KCl and 10 μ mol/L norepinephrine. Arteries were then washed and stimulated with 10 nmol/L AngII. Data represented as percentage of maximal contraction achieved by AngII stimulation (**A**) and AngII response curves as a function of time (**B**). Statistical analysis is presented as means $\pm$ SEM. (WT, $n = 3$; OVE26, $n = 6$; $EP1^{-/-}$, $n = 4$; OVE26 $EP1^{-/-}$, $n = 6$). * $P < 0.05$, *** $P < 0.001$.

generation. In addition, TEM revealed that DN-induced GBM and podocyte foot process ultrastructural damage were significantly reduced in OVE26*EP1*^{-/-} mice, suggesting a direct detrimental effect of PGE₂/EP1 signaling on podocyte health and glomerular filtration barrier integrity. Thus, activation of the podocyte EP1 receptor may lead to morphologic changes while providing additional Gq-signaling input, promoting a pro-oxidant environment that leads to further filtration barrier damage. Whether the reduced albuminuria seen in diabetic *EP1*^{-/-} mice was primarily governed by a loss of podocyte EP1 activity, thereby directly preventing damage to this final layer of the filtration barrier, will require further investigation.

Although filtration barrier injury likely accounts for most of the urinary albumin content in DN, the PT may also play a role in the early stages.^{66,67} PT-mediated albumin reabsorption occurs at the level of the brush border where the megalin-cubilin endocytic protein complex is abundantly expressed. In healthy individuals, minute amounts of albumin (600 mg/d) leak through the glomerulus and reach the PT, yet <30 mg is detected in the urine, which implies that the PT reabsorbs 95% of filtered albumin. In a small T1DM study cohort, microalbuminuria was associated with increased megalinuria and cubilinuria, possibly due to increased matrix metalloprotease-induced shedding in the PT lumen.⁶⁸ Furthermore, diabetic rats have decreased PT-mediated albumin reabsorption, an effect that can be blocked by antagonizing RAAS activation.^{52,69} We measured cortical megalin expression in our T1DM mouse cohorts to assess the impact of EP1 deletion on PT integrity. Our results reveal decreased immunodetectable renal megalin for both diabetic models. EP1 deletion prevented megalin protein down-regulation but not mRNA expression, thereby suggesting posttranslational regulation of this gene product. It remains unclear whether activation of the EP1 on PT cells directly affects megalin expression or instability, thereby decreasing albumin reabsorption, or if increased glomerular albumin leakage coupled with toxic luminal albumin concentrations may result in megalin down-regulation and associated PT dysfunction. Of interest, activation of the functionally similar, Gq-coupled AT1 receptor has been found to down-regulate PT-megalin expression.⁷⁰ In these studies, AngII infusion reduced both megalin expression and albumin endocytosis in the PTs of rats with streptozotocin-induced T1DM,⁶⁹ an effect that was prevented by angiotensin-converting enzyme inhibition or AngII receptor blockers. Interestingly, new evidence suggests the involvement of the COX/PGE₂/EP1 pathways in modulating the RAAS system. PGE₂/EP1 activation facilitates AngII-mediated oxidative stress and endothelial damage in the cerebral vasculature⁷¹ and is a major player in hypertensive renal damage.^{32,47,72,73} In agreement with these studies, our work finds that EP1 deletion reduced AngII-mediated contractility of isolated mesenteric arteries and attenuated AngII-stimulated oxidative stress in cultured podocytes. The precise molecular mechanism that accounts

for such interactions awaits investigation. Moreover, because the present studies were conducted using mesenteric arteries, whether such receptor interactions occur in glomerular vessels remains unknown. However, it is not unreasonable to speculate that EP1/AT1-stimulated vasoconstriction of the efferent arteriole could contribute to intraglomerular capillary pressure elevations, thereby contributing to filtration barrier damage in DN. Despite these observations, the effect of EP1 receptor deletion on systolic BP pressure was not evident in our studies because it did not differ from that of wild-type mice with or without diabetes. Taken together, if EP1/AT1 dependency represents a general phenomenon, occurring wherever these two receptors are co-expressed, our data would suggest that inhibiting PGE₂/EP1 signaling may complement existing RAAS blockade treatments, thereby conferring additional renoprotection in DN.

Conclusion

Abolishing EP1 signaling is protective against the onset and progression of early DN in mice with T1DM because it reduces the extent of renal structural and functional damage. It remains unclear whether PGE₂/EP1 signaling is detrimental to a specific resident renal cell type because both glomerular and tubular compartments benefitted from abrogated EP1 activation. Further studies should be undertaken to fully elucidate the role of the EP1 receptor in other renal compartments, including the vasculature in DN. Targeting the renal EP1 may represent a worthy therapeutic target to circumvent adverse effects associated with current COX-modulating drugs.

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