Protective Role of Small Pigment Epithelium Derived Factor (PEDF) Peptide in Diabetic Renal Injury

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Abstract

Pigment epithelium derived factor (PEDF) is a multifunctional protein with antiangiogenic, anti-oxidative and anti-inflammatory properties. PEDF is involved in the pathogenesis of diabetic retinopathy, but its direct role in the kidneys remains unclear. We hypothesize that a PEDF fragment (P78-PEDF) confers kidney protection in diabetic nephropathy (DN).

The localization of the full length PEDF protein were determined in DBA mice following multiple low doses of streptozotocin. Using immunohistochemistry, PEDF was localized in the kidney vasculature, interstitial space, glomeruli, tubules and renal medulla. Kidney PEDF protein and mRNA expression were reduced significantly in diabetic mice. Continuous infusion of P78-PEDF for 6 wks resulted in protection from DN as indicated by reduced albuminuria and blood urea nitrogen, increased nephrin expression, decreased kidney macrophage recruitment and inflammatory cytokines, and reduced histological changes compared with vehicle-treated diabetic mice. In vitro, P78-PEDF blocked the increase in podocyte permeability to albumin and disruption of the actin cytoskeleton induced by puromycin aminonucleoside (PAN) treatment.

These findings highlight the importance of P78-PEDF peptide as a potential therapeutic modality in early phase diabetic renal injury.

Keywords: PEDF, Diabetic Nephropathy, Podocytes
Introduction

Diabetes mellitus (DM) is a leading cause of morbidity and mortality in the United States. DM is often complicated by micro- and macrovascular involvement which contribute to damage to one or more target organs. Diabetic nephropathy (DN) is a well-known microvascular complication of diabetes mellitus and is responsible for 40-50% of all cases of end stage renal disease (ESRD) in the U.S. adult population (13, 35). The rate of progression of DN towards ESRD is influenced by complex interactions between genetic predisposition, dietary and lifestyle factors as well as therapeutic interventions. Available therapeutic options directed at delaying the progression of DN include intensive blood glucose control, improved blood pressure control, interruption of the renin angiotensin aldosterone system along with dietary modification and cholesterol-lowering agents (for review please see: (1)). Despite aggressive multifactorial interventions, DN remains the single leading cause of ESRD in the United States. Therefore, more effective approaches are urgently needed.

The pathogenesis of DN involves multiple processes including: inflammation, angiogenesis, oxidative injury and podocyte structural and functional abnormalities. Pigment epithelium derived factor (PEDF) is a multifunctional, pleiotropic secretory glycoprotein with antiangiogenic, anti-oxidative and anti-inflammatory properties (31, 33). The PEDF protein was first identified in the eye (34) but is also present in other tissues, including the kidney (26). The human PEDF gene encodes a 418-amino acid protein (9) and shows strong conservation across phyla (32). PEDF acts via multiple high affinity ligands and cell receptors, although the mechanisms are not clear. Previous reports demonstrated that PEDF is reduced in diabetic retinopathy and that systemic or
local delivery of recombinant PEDF protein or viral vector-mediated PEDF gene therapy successfully inhibited retinal neovascularization and reduced retinal vascular permeability in diabetic animals (22). However, the direct role of PEDF in the diabetic kidneys is not completely clear. Since PEDF is a 50 kDa protein, this may limit its utility as a therapeutic target. Recent evidence has suggested that fragments of PEDF are bioactive. In particular, a 44 amino acid (AA 78-121; P78-PEDF) peptide shows excellent bioactivity in several reports (15, 22). Thus, it is important to identify the direct role of PEDF and/or P78-PEDF peptide in the setting of DN.

The current study tested the hypothesis that a PEDF fragment (P78-PEDF) confers kidney protection in DN. We found that STZ-induced Type 1 diabetic mice are protected from albuminuria and display reduced histopathological changes associated with DN and reduced macrophage infiltration into the kidney when treated with P78-PEDF peptide. In addition, P78-PEDF peptide had a direct effect to restore podocyte structural and functional integrity. These results provide evidence for P78-PEDF peptide as a novel therapeutic intervention in the treatment of early phase DN.
Materials and Methods

Diabetic mouse model. Experiments were conducted in male 6-week-old DBA/2J mice (The Jackson Laboratory, stock number 000671) and approved by Penn State University College of Medicine Institutional Animal Care and Use Committee. DBA/2J mice are recommended by the Animal Models of Diabetes Complications Consortium (AMDCC) as a nephropathy susceptible model of DN (10, 11). Type-1 diabetes mellitus was induced using multiple low doses of streptozotocin (STZ; Sigma, St. Louis, MO; 50 mg/kg body wt dissolved in lactated Ringers solution) via IP injection for 5 consecutive days. Establishment of diabetes mellitus was confirmed 5 days after the last dose of STZ injection by measuring random blood glucose levels (Accu-Chek glucometer, Boehringer Mannheim, Indianapolis, IN). All analyses were done from the left kidney except FACS analysis which was performed on the right kidney.

Drug delivery. P78-PEDF; a small PEDF peptide (15, 18, 22) (0.1 µg/g/day) or vehicle (PBS) were administered by continuous subcutaneous infusion beginning immediately after confirming the elevated blood glucose level via an osmotic mini-pump (model 2006, Alzet; Durect Corporation, Palo Alto, CA, USA) which deliver drug for 6 weeks after a single pump placement as described previously (5, 24, 27). The condition of mice and body weight were monitored daily following the pump implantation. All endpoint data were collected after 6 weeks of P78-PEDF treatment (13 weeks of age).

Blood pressure measurement. Mean arterial pressure (MAP) was measured using the Coda blood pressure system (Kent Scientific Corp, Torrington, Connecticut) (5, 14, 24).
Mice were allowed to rest quietly for 10 min at 26°C. All measurements were performed at the same time for all groups to prevent any diurnal variations.

Renal histopathology. Kidneys were fixed in 4% paraformaldehyde, embedded in paraffin, and 3-µm sections were cut. Sections were stained with periodic acid-Schiff (PAS) stain and all glomeruli were examined individually at 400x in a blinded manner and scores were averaged. All images were obtained with an Olympus BX51 microscope and DP71 digital camera using cellSens Standard 1.6 imaging software. Images were taken with 100x (oil) objective (total magnification of 1000x). Semiquantitative scores (0 – 4) were assigned based on the masked reading, as previously described (5, 24, 41). Briefly, each glomerulus on a kidney single section was graded from 0 to 4, where 0 represents no lesion, and 1, 2, 3, and 4 represent mesangial matrix expansion or sclerosis, involving ≤25, 25 to 50, 50 to 75, or >75% of the glomerular tuft area, respectively.

Glomerular macrophage staining. Immunohistochemistry for macrophages was performed using rat anti-mouse Mac-2 antibody (clone M3/38; Cedarlane, Burlington, NC) on paraffin sections as described previously (5, 24).

PEDF Immunocytochemistry. Kidney samples were fixed in 4% (v/v) PFA, cryopreserved in 30% sucrose, embedded in OCT, and 10 um cryosections obtained. Non specific binding sites on the samples were blocked for 30 min with 0.5% BSA. The polyclonal antibodies; PEDF (1:50 dilution) and β-actin (1:50 dilution; Millipore,
Temecula, CA) were diluted in PBS containing 0.5% BSA. Cy3- or FITC- conjugated goat anti-rabbit IgG’s diluted 1:1000 in 5% BSA/PBS were used as secondary antibodies. DAPI (1:5,000 – 5mg/ml solution) was applied to immunolabeled tissues for 10 min then samples were mounted on glass slides using Slowfade gold antifade reagent (Invitrogen). Fluorescence labeling was evaluated by confocal microscopy (Olympus; Fluoview 1000).

**Analysis of kidney macrophage content by FACS.** Kidney macrophage content was determined by flow cytometry at the end of experiments as described previously (5, 6, 8). In brief, kidneys were extracted, minced, digested, and then passed through a filter and a cotton wool column. Fresh kidney suspensions were incubated with anti-mouse CD45-FITC (30-F11; eBioscience, San Diego, CA) for 30 min on ice. Kidney macrophages were then identified using allophycocyanin (APC)-labeled rat anti-mouse F4/80 (BM8; eBioscience) and phycoerythrin (PE)-labeled rat anti-mouse CD11b (M1/70; eBioscience). All samples were treated with anti-mouse CD16/CD32 (2.4G2) to block nonspecific FcR binding and 7-AAD to eliminate dead cells (Invitrogen, Carlsbad, CA). Counting beads (Caltag, Carlsbad, CA) were used to determine the total number of CD45+ cells per gram of kidney tissue. Subsequent flow cytometry data acquisition was performed on LSR2 (Becton Dickinson, San Jose, CA). Data were analyzed by Flowjo software 8.7 (Tree Star, Ashland, OR). All the antibodies were purchased from eBioscience (San Diego, CA).

**Podocyte BSA Filter Assay.** Collagen-coated Transwell-Col PTFE filters (3 µm pore; Corning, New York, NY) were seeded with $1 \times 10^4$ podocytes per filter and cultured
under differentiating conditions for 14 d. Cells were then incubated with vehicle or 100 μg/ml puromycin aminonucleoside (PAN, Sigma) with or without P78-PEDF peptide (100 nM) for 24 h at 37°C. The upper compartment was then refilled with 0.5 ml of RPMI 1640 and the lower compartment with 1 ml of BSA medium (RPMI 1640 supplemented with 40 mg/ml BSA). Total protein concentration in the upper compartment was determined at 6 h using a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) as described previously (7).

**Immunofluorescence and Confocal Microscopy.** Differentiated podocytes grown on collagen-coated glass coverslips were incubated with FITC-conjugated phalloidin (Sigma) as described previously (7). All specimens’ fields were selected randomly and examined using a Zeiss LSM 510-UV confocal microscope.

**Quantitative real-time RT-PCR.** Total RNA was extracted from kidneys and podocytes using RNeasy Mini Kit (Qiagen, Gumbh, Hilden, Germany). Single-strand cDNA was synthesized using Superscript First Strand cDNA Synthesis Kits (Invitrogen, CA) for two-step real-time RTPCR. Gene-specific primers for nephrin, PEDF and GAPDH were designed using Beacon Designer Probe/Primer Design Software (OligoPerfect Designer, Invitrogen, Carlsbad, CA). Quantitative real-time PCR was performed using Bio-Rad iQ5 system (Hercules, CA). Reactions were performed in duplicate, and threshold cycle numbers were averaged. Results were normalized to GAPDH measured in parallel (4).
Western Blot. Samples were homogenized in lysis buffer and soluble protein extracted by centrifugation. Thirty microgram aliquots of extracted proteins were separated in 10% SDS polyacrylamide gels and electro-transferred for 2 hr at 0.3A onto nitrocellulose membranes (Bio-Rad, Laboratories Inc. Hercules, CA). Non-specific antigen binding sites were blocked with 5% non-fat dried milk and transblots incubated overnight at 4°C with 1:500 PEDF polyclonal antibody. PEDF antibody specificity was previously confirmed (21). Membranes were then washed, exposed for 1 hr to horseradish peroxidase (HRP)-conjugated affinity purified goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA), and HRP activity assessed using the Pierce enhanced chemiluminescent western blotting substrate (ECL; Thermo scientific. Rockford, IL) followed by exposure to Kodak Scientific Imaging X-OMAT LS Film (Carestream Health, Inc. Rochester, NY). Blots were stripped and reprobed for β-actin. PEDF results were normalized to β-actin.

Glomerular endothelial cell lines: Mouse glomerular endothelial cells (kindly provided by Dr. Michael Madaio, Medical College of Georgia) were cultured as described previously (3). Briefly, frozen cells were grown under permissive conditions to propagate (in the presence of 50 U/ml gamma-interferon at 33°C) in DMEM/F12 media containing 10% fetal bovine serum, penicillin(100 U/mL), streptomycin (100 µg/mL), glutamine (2 mmol/L) in collagen-coated plate. Cells then were grown under restrictive conditions for 14 days (absence of gamma-interferon at 37°C in 95% air/5% CO2) and allowed to differentiate in the presence of normal glucose (11 mM) or high glucose (33 mM) media. After 14 days, cells were subjected to western blot as described above.
**Analytical methods.** Urinary albumin excretion was measured by ELISA using Albuwell M (Exocell, Philadelphia, PA) as described previously (4, 5, 24). Urine creatinine was determined using a Creatinine Liquid Reagens Assay kit (Diazyme Laboratories, Poway, CA) as described previously (5, 24). BUN was measured using VITROS DT60II chemistry slides (Ortho-Clinical Diagnostics, Rochester, NY) as described previously (5, 24). Urine TNF-α was measured by ELISA (eBioscience) as we described previously (5). Vascular endothelial growth factor (VEGF) level was determined using the Affymetrix Procarta Cytokine assay (Panomics. Fremont, CA) in conjunction with the Bio-Plex instrument (Bio-Plex 200, Bio-Rad Laboratories Inc. Hercules, CA) as we described previously (21). Body composition was determined using LF90 Minispec Time Domain Nuclear Magnetic Resonance Spectrometer (Bruker Optics, Billerica MA) as described previously (5, 24).

**Statistical analysis.** Comparisons between groups were examined by using the SPSS version 19.0 software (SPSS, Chicago, IL) program. Data are expressed as mean ± SEM. One-way ANOVA was used when more than two groups were compared, and significance of observed differences among the groups was evaluated with a least significant difference post hoc test. Statistical significance was identified at $p< 0.05$. 
Results

Localization of PEDF in mouse kidneys. The exact localization of PEDF in the kidney is not clear. Therefore, we first assessed the localization of PEDF in the kidneys under normal conditions. Using a PEDF specific antibody (21), we identified PEDF expression in the mouse kidneys (Fig. 1); mainly in the vasculature, interstitial spaces, glomeruli, medulla and tubular epithelial cells.

PEDF protein and mRNA expression is reduced in diabetic kidneys. Next, we determined PEDF expression in diabetic kidneys. Our data show that PEDF protein (Fig. 2A) and mRNA (Fig. 2B) expressions are markedly reduced in diabetic kidneys after 6 weeks of STZ-induced diabetes mellitus. We also confirmed the expression of PEDF in glomerular endothelial cells in vitro; and noted that PEDF expression was significantly reduced after growth in high glucose media for 14 days (Fig. 2C). Although total PEDF expression was reduced in diabetic mice, the localization within the kidney was similar to control mice (data not shown).

P78-PEDF peptide administration reduces characteristics of diabetic nephropathy. Since PEDF is reduced in diabetic kidneys, we questioned whether restoring PEDF using P78-PEDF peptide ameliorates diabetic renal injury. Towards this goal, we continuously infused the P78-PEDF peptide or vehicle into diabetic mice for 6 weeks. As shown in Table 1, vehicle-treated diabetic mice had increased blood glucose levels, decreased body weight, decreased kidney weight, increased kidney weight/body weight ratio, and reduced fluid composition compared to normal mice. P78-PEDF peptide administration to diabetic mice significantly increased body weight and reduced
kidney weight/body weight ratio without affecting other measurements compared to vehicle-treated diabetic group. Importantly, treatment with P78-PEDF peptide did not affect blood pressure, blood glucose or fluid composition compared to vehicle-treated diabetic mice.

**P78-PEDF peptide administration reduces albuminuria and blood urea nitrogen in diabetic mice.** To determine if PEDF deficiency is associated with diabetic renal injury, we measured 24-hr urine albumin excretion (UAE), albumin/creatinine ratio and blood urea nitrogen (BUN) as indicators of renal injury in diabetic mice treated with vehicle or P78-PEDF peptide. Vehicle-treated diabetic mice had a significant increase in albuminuria (**Fig. 3A**), albumin/creatinine ratio (**Fig. 3B**), and BUN (**Table 1**) compared to normal mice after 6 weeks of diabetes mellitus. These effects were significantly reduced in diabetic mice treated with P78-PEDF peptide for 6 weeks.

**P78-PEDF peptide administration decreases macrophage recruitment in STZ-induced diabetic mice.** To determine whether the reduction of PEDF is critical for macrophage infiltration in DN, we show the distribution and quantitation of macrophages in kidneys by immunohistochemistry (Mac-2 positive macrophages) (**Fig. 4**). Vehicle-treated diabetic mice showed increases in glomerular macrophages compared to normal mice. In contrast, P78-PEDF peptide treated diabetic mice had reduced glomerular macrophage recruitment. Similar results were obtained when total kidney macrophages (identified as CD11b<sup>+</sup>F4/80<sub>low</sub>) were measured by FACS. Kidneys of vehicle-treated diabetic mice had a significantly greater number of macrophages
compared with normal mice ($24.1\pm2.7 \times 10^4$ vs. $5.2\pm0.8 \times 10^4$ macrophages/gm kidney tissue, $p<0.0001$). In contrast, kidneys of P78-PEDF peptide treated diabetic mice had significantly reduced numbers of macrophages ($16.7\pm2.4 \times 10^4$ macrophages/gm kidney tissue, $p<0.05$) compared to vehicle-treated diabetic mice at 6 weeks of STZ-induced diabetes mellitus.

**P78-PEDF peptide administration decreases kidney and urinary cytokines in diabetic mice.** Increased inflammatory cytokines is a major feature of and an important predictor of DN (4, 16). Therefore, we further assessed the anti-inflammatory effect of P78-PEDF peptide treatment in diabetic mice (Fig. 5). Urinary tumor necrosis factor (TNF)-α excretion (Fig. 5A) and kidney VEGF (Fig. 5B) were significantly increased in the vehicle-treated diabetic mice at 6 week of diabetes mellitus ($p<0.01$) compared to normal mice. In contrast, P78-PEDF peptide significantly decreased urinary TNF-α excretion and kidney VEGF in diabetic mice ($p<0.05$ vs. vehicle treated mice).

**P78-PEDF peptide administration decreases renal histopathological changes in STZ-induced diabetic mice.** Periodic acid-Schiff (PAS) staining of kidney sections (Fig. 6) showed increased glomerular cellularity and mesangial expansion (score: $0.9\pm0.03$ vs. $0.3\pm0.01$, $p<0.0001$) in vehicle-treated STZ-diabetic mice vs. normal mice; respectively. P78-PEDF peptide treatment resulted in significantly reduced glomerular changes (scores: $0.4\pm0.06$; $p<0.005$) compared to vehicle-treated diabetic mice.
**P78-PEDF peptide administration increases renal PEDF expression in STZ-induced diabetic mice.** We also determined the effect of P78-PEDF peptide treatment on PEDF expression in diabetic kidneys. Our data show that PEDF protein expression is markedly reduced in diabetic kidneys after 6 weeks of STZ-induced diabetes mellitus. Interestingly, P78-PEDF peptide treatment significantly increased PEDF protein expression in diabetic mice (Fig. 7). Immunohistochemistry for PEDF did not reveal any changes in PEDF cellular localization in peptide-treated mice (data not shown).

**P78-PEDF peptide administration regulates podocyte-specific proteins; Nephrin.** To determine the possible mechanisms by which P78-PEDF peptide reduced albuminuria and mediates renal tissue protection, we examined its effect on podocytes. At 6 weeks of diabetes mellitus, nephrin (a podocyte-specific protein) mRNA was reduced in kidneys from vehicle-treated diabetic mice, as expected. P78-PEDF treated diabetic mice had markedly increased expression of nephrin compared to vehicle treated controls, but was unchanged from normal ($p<0.05$) (Fig. 8).

**P78-PEDF peptide attenuates puromycin aminonucleoside (PAN)-Induced increase in podocyte permeability in vitro.** To confirm the direct functional effect of P78-PEDF peptide on podocytes, we determined its effect on podocyte permeability by measuring the transepithelial passage of bovine serum albumin (BSA) across differentiated podocytes grown on Transwell chambers as described previously (7). As shown in Fig. 9, differentiated podocytes treated with PAN significantly increased albumin passage across podocytes ($p<0.005$ compared to normal).
P78-PEDF peptide treatment completely blocked the effect of PAN on podocyte permeability to albumin (p<0.05). P78-PEDF peptide treatments also resulted in preservation of podocyte structural integrity and prevention of the marked disruption and organization of the actin cytoskeleton produced by treatment with PAN and preservation of podocyte actin mRNA expression (Fig. 10).
Discussion

PEDF has well-established anti-angiogenic, anti-oxidative and anti-inflammatory actions in the eye (31, 33), yet its role in diabetic kidney injury is not completely clear. This study shows that a small bioactive peptide fragment of PEDF (P78-PEDF) mediates renal tissue protection as evidenced by a reduction in albuminuria, BUN, histopathological changes, kidney macrophage recruitment, and inflammatory cytokines during diabetes mellitus. Furthermore, P78-PEDF preserved expression of podocyte structural protein (nephrin) during diabetes mellitus in vivo and directly preserved podocyte structural and functional integrity in vitro. These findings reveal an important role for P78-PEDF peptide and/or other PEDF peptides in the pathogenesis of early phase DN. P78-PEDF could provide a new therapeutic modality for treating diabetic patients.

PEDF protein is expressed in several tissues and cells including the postnatal kidney (26). However, the exact localization of PEDF in the kidney is not known. In our experiment, we used a specific antibody to clearly identify PEDF expression in the mouse kidney. Our data show that PEDF is expressed mainly in the kidney vasculature, interstitial spaces, glomeruli, medulla and tubular epithelial cells. We also were able to detect the expression of PEDF in glomerular endothelial cells in vitro in normal glucose media; an effect significantly reduced using high glucose media. We further questioned whether PEDF is altered in diabetes mellitus. Our data show that both PEDF protein and mRNA expression are markedly reduced in diabetic kidneys. These data are consistent with a previous report of decreased PEDF protein and mRNA expression in diabetic rat kidney (38).
To examine the role of PEDF in DN, we continuously infused a small bioactive fragment of PEDF, P78-PEDF peptide, in this mouse model of type-1 diabetes mellitus. P78-PEDF peptide has been recently characterized (22) and shown to have excellent bioactivity. Unlike P78-PEDF peptide, other approaches have mainly focused on PEDF delivery using viral vectors (39, 40). These approaches have problems associated with inflammation to viral components, difficulty regulating the level of PEDF produced, and concerns over the rapidity of onset and longevity of PEDF expression (9). For example, adenoviral delivery of PEDF failed to maintain the reduction in albuminuria in diabetic rats after 5 weeks of diabetes mellitus (40). Given these concerns, we investigated the effects of P78-PEDF peptide on diabetic kidney dysfunction, specifically glomerular histopathological changes and macrophage recruitment. P78-PEDF peptide significantly ameliorated diabetic albuminuria and was associated with reduced kidney macrophage infiltration and glomerular pathology. Taken together, our results provide support for P78-PEDF peptide as a therapeutic modality for the treatment of early phase DN. Additional study is needed to explore the effect of P78-PEDF peptide treatment in late stage DN.

The renal protective effect of P78-PEDF peptide correlates with a significant reduction in kidney macrophage infiltration. Whether the reduction in macrophage recruitment is mediated directly by P78-PEDF peptide or indirectly by reducing diabetic renal injury will require additional further studies. Infiltrating macrophages release lysosomal enzymes, nitric oxide, reactive oxygen species, transforming growth factor-beta, vascular endothelial growth factor and cytokines such as TNF-α, interleukin-1 and interferon (IFN)-γ (30) which could play a pivotal role in the development and
progression of DN. We have shown previously that urinary TNF-α is increased in a mouse and rat model of type-1 diabetes mellitus (4, 5). The current study confirms these results and shows that P78-PEDF peptide significantly reduced the increase in urinary TNF-α. TNF-α is produced mainly by monocytes/macrophages and has been associated with increasing vascular endothelial permeability in diabetes mellitus (25). Our data also show reduced kidney VEGF with P78-PEDF peptide treatment confirming the observation that PEDF counteracts the effects of VEGF (20). The role of VEGF in renal physiology and physiopathology is controversial. Under physiological conditions, VEGF maintains glomerular endothelial integrity. In certain conditions, such as hypertension, inhibition of VEGF is associated with severe glomerulosclerosis, mesangial expansion and albuminuria (2). In contrast, increased VEGF can lead to glomerular hypertrophy and proteinuria (19). Indeed, overexpression of VEGF in podocytes of transgenic mice is associated with thickened glomerular basement membrane and proteinuria (36, 37); a cardinal feature of DN. In animal models of DN, VEGF levels are elevated (12) and inhibition of VEGF using a small molecule inhibitor of VEGFR (23) significantly ameliorated albuminuria (29). Likewise, ruboxistaurin has been shown to attenuate the effect of VEGF and progression in animal studies of DN (17). Additional study is needed to explore the direct role of P78-PEDF peptide on kidney VEGF following diabetes mellitus. Interestingly, P78 PEDF peptide reduced retinal IFN-γ, TNF-α, loss of retinal ganglion cell layer (RGL) and increased the thickness of the inner plexiform layer in our diabetic mice compared to the vehicle treated group (data not shown). P78-PEDF peptide also resulted in less mesangial expansion and glomerular hypercellularity in diabetes mellitus, indicating a possible
contribution of PEDF reduction to the initiation and/or progression of diabetic renal fibrosis.

Renal PEDF expression was reduced in STZ diabetic mice. However, our data show that P78-PEDF peptide treatment increased endogenous PEDF expression in diabetic mice. It is possible that some of the beneficial actions of P78-PEDF we observed in DN could be mediated by the increase in endogenous PEDF. Studies in PEDF deficient mice will be needed to address this possibility. Another possible interpretation is that the reduction in endogenous PEDF expression is a result of DN. These two possibilities are not mutually exclusive, as a reduction in PEDF could contribute to the pathogenesis of DN and, in turn, DN could decrease PEDF, leading to further disease progression. Additional studies are needed to confirm this hypothesis.

The mechanism by which P78-PEDF peptide mediates protection in STZ-induced DN is not known; however, we speculate that P78-PEDF peptide may have a direct effect on podocytes. This hypothesis is based on 2 important findings. First, we show that P78-PEDF peptide restored nephrin expression in diabetic mice. Second, our previous publication (26) indicates a colocalization of PEDF with synaptopodin in the kidney. Podocytes play a key role in the maintenance of the glomerular filtration barrier (28), and normal podocyte function is intimately linked to its complex cytoskeletal architecture. Because the expression of nephrin is limited to podocytes, we questioned whether the protective effect of P78-PEDF peptide in reducing proteinuria may, in part, be due to direct effects on podocytes. Toward this goal, we examined the direct effect of P78-PEDF peptide on the maintenance of podocyte functional and structural integrity in vitro. In the current study, we used puromycin as a non-specific insult since high
glucose insults do not induce podocyte injury *in vitro*. Our data show that PAN disrupts the highly organized actin cytoskeleton and increased the transepithelial passage of BSA. Treatment of podocytes with P78-PEDF peptide restored both the architecture of the podocyte cytoskeleton and its functional integrity, suggesting that the observed effect of P78-PEDF peptide *in vivo* may be due to direct effects on podocytes. However, additional experiments are needed to demonstrate the direct effect of P78-PEDF peptide in podocytes *in vivo*.

In conclusion, our study demonstrates that a PEDF fragment (P78-PEDF) confers kidney protection in early phase DN. This conclusion is based on two novel observations: First, we demonstrated for the first time a beneficial effect of P78-PEDF peptide in animal models of DN. Second, we showed that P78-PEDF peptide has a direct effect to maintain podocyte functional and structural integrity. Results of this study may ultimately result in novel therapeutic interventions using P78-PEDF peptide in the treatment of diabetic kidney disease.
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Author Contributions: ASA; wrote manuscript, researched data and developed the idea, TG; researched data, AG; researched data, HY; researched data, YL; researched data, TKC; researched data and reviewed manuscript, WBR; researched data and contributed to discussion, JT; researched data and developed the idea.
Disclosure

None
References


Tables and legends

Table 1: Effects of P78-PEDF peptide administration on diabetic mice.
Data are mean ± SEM. SBP: systolic blood pressure. *p<0.0001, **p<0.001 compared to non-diabetic group; *p<0.005, **p<0.0001 compared to diabetes + vehicle group (n=12-14 mice per group).

Figure 1: Localization of PEDF in the mouse kidney. Immunofluorescence staining for PEDF on representative kidney section in normal mice.

Figure 2: PEDF expression in vivo and in vitro. (A-B) Western-blot and RT-PCR were performed to detect PEDF expression in kidney lysates from indicated groups of mice. A) Western blot analysis of total kidney PEDF expression (n=6 each group). Semi-quantification of PEDF expression was performed by densitometry followed by normalization to β-actin. B) Quantitative real-time PCR of total kidney PEDF expression (n=5 each group). Data were normalized to GAPDH. Open bar, normal; black-filled bar, diabetes. Results are means ± SEM. C) Western blot analysis of PEDF in glomerular endothelial cells grown in normal or high glucose media (n=6 each group). Semi-quantification of PEDF expression was performed by densitometry followed by normalization to β-actin. Open bar, normal glucose; black-filled bar, high glucose. Results are means ± SEM.

Figure 3: P78-PEDF peptide attenuates diabetic renal injury. Diabetic mice were treated with P78-PEDF peptide or vehicle via osmotic minipump for 6 weeks. Urine was
collected for measurement of UAE (A) or albumin/creatinine ratio (B). Open bar, normal group; black-filled bar, vehicle-treated diabetic groups; grey-filled bar, P78 PEDF-treated groups. Results are means ± SEM.

**Figure 4:** P78-PEDF peptide reduces macrophage infiltration in diabetic mice. Mac-2-positive macrophages in glomeruli were identified by immunohistochemical staining after 6 weeks in normal, vehicle-treated diabetic and P78-PEDF-treated mice. Images are representative of 40 fields from 12-14 mice in each group. Red arrow indicates stained macrophages.

**Figure 5:** P78-PEDF peptide reduces inflammatory cytokines in diabetic mice. Kidney lysate and urine were collected for measurements of VEGF and TNF-α; respectively after 6 weeks of diabetes mellitus. Open bar, normal group; black-filled bar, vehicle-treated diabetic groups; grey-filled bar, P78-PEDF-treated groups. Results are means ± SEM.

**Figure 6.** P78-PEDF peptide reduces histological changes in diabetic mice. Sections were stained with PAS and all glomeruli were graded individually at 400x magnification. Normal mice have normal amounts of delicate mesangium supporting capillaries. In vehicle-treated diabetic mice, PAS positive matrix expands the glomerular tuft by less than 25%. Mice treated with P78-PEDF have glomeruli indistinguishable from normal. Images were taken with 100x (oil) objective with a total magnification of 1000x. Images are representative of from 12-14 mice in each group.
Figure 7: P78-PEDF peptide increases PEDF expression in diabetic mice. Western-blot was performed to detect PEDF expression in kidney lysates from indicated groups of mice. Semi-quantification of PEDF expression was performed by densitometry followed by normalization to β-actin. Open bar, normal group; black-filled bar, vehicle-treated diabetic groups; grey-filled bar, P78-PEDF-treated groups. Results are means ± SEM.

Figure 8: P78-PEDF peptide regulates podocyte-specific nephrin expression in diabetic mice. Quantitative real-time RT-PCR was performed on whole kidney total RNA isolated after 6 weeks of diabetes. Expression of nephrin mRNA was normalized to GAPDH and data were calculated as expression relative to control. Open bar, normal group; black-filled bar, vehicle-treated diabetic groups; grey-filled bar, P78-PEDF-treated groups (n=11-12 mice per group). Results are means ± SEM.

Figure 9: P78-PEDF peptide regulates podocyte permeability in vitro. Transepithelial permeability of differentiated podocytes to BSA was measured as described previously. After 14 days of culture on Transwell filter chambers (3-μm pore) at 37°C, podocytes were pretreated with vehicle or P78-PEDF peptide (100 nM) for 1 h and then exposed to PAN (100 μg/ml) for 24 h. The lower chambers were then filled with BSA-containing medium, and the upper chambers were sampled at 6 h (n = 6 each group). Data are means ± SEM.
Figure 10: P78-PEDF peptide regulates podocyte actin cytoskeleton in vitro. After 14 days of culture at 37°C, podocytes were pretreated with vehicle or P78-PEDF peptide (100 nM) for 1 h and then exposed to PAN (100 μg/ml) for 24 h. Confocal images of differentiated murine podocytes in normal, podocytes treated with PAN alone, or podocytes treated with PAN + P78-PEDF peptide. Bar graph: Quantitative real-time RT-PCR was performed on differentiated podocytes. Expression of actin mRNA was normalized to GAPDH and data were calculated as expression relative to control. Results are means ± SEM. N; normal podocytes, N+P; normal podocytes treated with P78-PEDF peptide, PAN; podocytes treated with puromycin aminonucleoside, PAN+P; podocytes treated with puromycin aminonucleoside and P78-PEDF peptide (n = 4 each group).
### Table 1: Effects of P78-PEDF peptide administration on diabetic mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Non-diabetic</th>
<th>Diabetes+vehicle</th>
<th>Diabetes+P78-PEDF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal #</td>
<td>13</td>
<td>14</td>
<td>12</td>
</tr>
<tr>
<td>Blood glucose (mg/dl)</td>
<td>135±5</td>
<td>471±9*</td>
<td>458±21*</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>27±0.7</td>
<td>20±0.5*</td>
<td>23±0.5*</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>126±3</td>
<td>123±5</td>
<td>124±5</td>
</tr>
<tr>
<td>Kidney weight (mg)</td>
<td>281±8</td>
<td>245±4**</td>
<td>236±6*</td>
</tr>
<tr>
<td>Kidney weight/body weight (mg/g)</td>
<td>10±0.2</td>
<td>12±0.2*</td>
<td>10±0.2**</td>
</tr>
<tr>
<td>% fluid</td>
<td>7.6±0.09</td>
<td>6.1±0.12*</td>
<td>6.1±0.21*</td>
</tr>
<tr>
<td>Plasma BUN (mg/dl)</td>
<td>16±1</td>
<td>34±2*</td>
<td>21±2**</td>
</tr>
</tbody>
</table>

Data are mean ± SEM. SBP: systolic blood pressure. *p<0.0001, **p<0.001 compared to non-diabetic group; *p<0.005, **p<0.0001 compared to diabetes + vehicle group.
Figure 1

- **Medium renal artery**
- **Interstitium**
- **Glomerulus**
- **Medulla**
- **Tubules**

**Color Code**
- Green: PEDF
- Blue: DAPI
- Red: Actin
Figure 2

A. Kidney PEDF protein (fold Δ)

B. Kidney PEDF mRNA (fold Δ)

C. Glomerular Endothelial PEDF protein (fold Δ)

PEDF 50 kDa
β-Actin 42 kDa

50 kDa
42 kDa
Figure 4

Normal

Diabetes + vehicle

Diabetes + P78-PEDF
Figure 5

A

Urine TNF-α (pg/24 hrs)

0 50 100 150 200 250

0.01 0.05

B

Kidney VEGF (pg/mg)

0 1000 2000 3000 4000 5000 6000 7000

0.01 0.05
Figure 6

Normal

Diabetes + vehicle

Diabetes + P78-PEDF
Figure 7

Kidney PEDF protein (fold Δ)

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Diabetes</th>
<th>Diabetes + P78-PEDF</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PEDF</strong></td>
<td><img src="#" alt="PEDF" /></td>
<td><img src="#" alt="PEDF" /></td>
<td><img src="#" alt="PEDF" /></td>
</tr>
<tr>
<td><strong>β-Actin</strong></td>
<td><img src="#" alt="β-Actin" /></td>
<td><img src="#" alt="β-Actin" /></td>
<td><img src="#" alt="β-Actin" /></td>
</tr>
</tbody>
</table>

50 kDa
42 kDa
Figure 9

BSA (mg/ml)

Filter  Normal  PAN  PAN+P78 PEDF peptide

0.005  0.05
Figure 10

Podocytes actin mRNA (fold change)

Normal  PAN  PAN + P78-PEDF peptide

Bar graph showing changes in podocytes actin mRNA (fold change) under different conditions: Normal, PAN, PAN + P78-PEDF peptide.