Protective role of small pigment epithelium-derived factor (PEDF) peptide in diabetic renal injury

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Diabetes mellitus is a leading cause of morbidity and mortality in the United States. Diabetes mellitus is often complicated by micro- and macrovascular involvement that contributes 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 toward ESRD is influenced by complex interactions between genetic predisposition, dietary and lifestyle factors, and 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, dietary modification, and cholesterol-lowering agents [for review 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, antioxidative, 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 significantly reduced in diabetic mice. Continuous infusion of P78-PEDF for 6 wk resulted in protection from diabetic neuropathy as indicated by reduced albuminuria and blood urea nitrogen, increased nephrin expression, decreased podocyte 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 treatment. These findings highlight the importance of P78-PEDF peptide as a potential therapeutic modality in early phase diabetic renal injury.

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Louis, MO) via intraperitoneal 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 performed on the left kidney except fluorescence-activated cell sorting (FACS) analysis, which was performed on the right kidney.

**Drug delivery.** P78-PEDF, a small PEDF peptide (15, 18, 21) (0.1 µg·g⁻¹·day⁻¹) or vehicle (phosphate-buffered saline; PBS) were administered by continuous subcutaneous infusion beginning immediately after confirming the elevated blood glucose level via an osmotic minipump (no. 2006; Alzet, Durect, Palo Alto, CA), which delivered drug for 6 wk 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 end point data were collected after 6 wk of P78-PEDF treatment (13 wk of age).

**Blood pressure measurement.** Mean arterial pressure was measured using the Coda blood pressure system (Kent Scientific, Torrington, CT) (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, all glomeruli were

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Fig. 1. Localization of pigment epithelium-derived factor (PEDF) in the mouse kidney. Immunofluorescence staining for PEDF on a representative kidney section in normal mice.
examined individually at 400× 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 the 100× (oil) objective (total magnification, 1,000×). Semiquantitative scores (0–4) were assigned on the basis of 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.

**Glyceraldehyde-3-phosphate dehydrogenase (GAPDH).** Open bar, normal; black-filled bar, diabetes. Results are means ± SEM. Two-tailed Student’s t test was used for comparisons between control and diabetes groups. The results are presented as means ± SEM. *p < 0.05.*

**Fig. 2. PEDF expression in vivo and in vitro.** 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). Semiquantification 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 glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Open bar, normal; black-filled bar, diabetes. Results are means ± SE. C: Western blot analysis of PEDF in glomerular endothelial cells grown in normal or high glucose media (*n* = 6 each group). Semiquantification of PEDF expression was performed by densitometry followed by normalization to β-actin. Open bar, normal glucose; black bar, high glucose. Results are means ± SE.
body specificity was previously confirmed (21). Membranes were then washed, exposed for 1 h to horseradish peroxidase (HRP)-conjugated affinity-purified goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), and HRP activity was assessed using a Pierce enhanced chemiluminescent Western blotting substrate (Thermo Scientific, Rockford, IL) followed by exposure to Kodak Scientific Imaging X-OMAT LS film (Carestream Health, 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. MichaelMadaio, 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 (INF-γ) at 33°C) in DMEM/F12 media containing 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 µg/ml), and glutamine (2 mmol/L) in collagen-coated plates. Cells then were grown under restrictive conditions for 14 days (absence of INF-γ 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 analysis 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). Blood urea nitrogen (BUN) was measured using VITROS DT60II chemistry slides (Ortho-Clinical Diagnostics, West Grove, PA), and HRP activity was assessed using a Pierce enhanced chemiluminescent Western blotting substrate.

**Statistical analysis.** Comparisons between groups were examined by using the SPSS version 19.0 software (SPSS, Chicago, IL) program. Data are expressed as means ± SE. 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 precise 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 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 wk 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

**Table 1. Effects of P78-PEDF peptide administration on diabetic mice**

<table>
<thead>
<tr>
<th>Group</th>
<th>Nondiabetic</th>
<th>Diabetes + Vehicle</th>
<th>Diabetes + P78-PEDF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal No.</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>

SBP, systolic blood pressure. Data are mean ± SE. *P < 0.0001; †P < 0.001 compared with the nondiabetic group; ‡P < 0.005; §P < 0.0001 compared with the diabetes + vehicle group (n = 12–14 mice per group).
mice, the localization within the kidney was similar to that of control mice (data not shown).

**P78-PEDF peptide administration reduces characteristics of diabetic nephropathy.** Because PEDF is reduced in diabetic kidneys, we questioned whether restoring PEDF using P78-PEDF peptide would ameliorate diabetic renal injury. Toward this goal, we continuously infused the P78-PEDF peptide or vehicle into diabetic mice for 6 wk. 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 with normal mice. P78-PEDF peptide administration to diabetic mice significantly increased body weight and reduced the kidney weight/body weight ratio without affecting other measurements compared with the vehicle-treated diabetic group. Importantly, treatment with P78-PEDF peptide did not affect blood pressure, blood glucose, or fluid composition compared with vehicle-treated diabetic mice.

**P78-PEDF peptide administration reduces albuminuria and BUN in diabetic mice.** To determine whether PEDF deficiency is associated with diabetic renal injury, we measured 24-h urine albumin excretion, albumin/creatinine ratio, and 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 with normal mice after 6 wk of diabetes mellitus. These effects were significantly reduced in diabetic mice treated with P78-PEDF peptide for 6 wk.

**P78-PEDF peptide administration decreases macrophage recruitment in STZ-induced diabetic mice.** To determine whether the reduction in 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 with 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⁺F4/80low) were measured by FACS. Kidneys of vehicle-treated diabetic mice had a significantly greater number of macrophages compared with normal mice (24.1 ± 2.7 × 10⁴ vs. 5.2 ± 0.8 × 10⁴ macrophages/g kidney tissue, P < 0.0001). In contrast, kidneys of P78-PEDF peptide-treated diabetic mice had significantly fewer macrophages (16.7 ± 2.4 × 10⁴ macrophages/g kidney tissue, P < 0.05) compared with vehicle-treated diabetic mice at 6 wk 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 DN and an important predictor of it (4, 16). Therefore, we further assessed the anti-inflammatory effect of P78-PEDF peptide treatment in diabetic mice (Fig. 5). Urinary TNF-α excretion (Fig. 5A) and kidney VEGF (Fig. 5B) were significantly increased in the vehicle-treated diabetic mice at 6 wk of diabetes mellitus (P < 0.01) compared with 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.** PAS staining of kidney sections (Fig. 6) showed increased glomerular cellularity and mesangial expansion (score, 0.9 ± 0.03 vs. 0.3 ± 0.01; P < 0.0001 in vehicle-treated STZ-diabetic mice vs. normal mice; respectively). P78-PEDF peptide treatment resulted in significantly reduced glomerular changes (score,
ROLE OF PEDF IN DIABETIC NEPHROPATHY

P78-PEDF peptide attenuates the 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 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 with 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 antiangiogenic, antioxidative, 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 and 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 patients with diabetes.

PEDF protein is expressed in several tissues and cells including the postnatal kidney (26). However, the precise 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 that was significantly reduced when 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 (21) 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 such as inflammation associated with 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 wk 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, and VEGF; and cytokines such as TNF-α, interleukin-1, and 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 VEGF receptor (23) sig-

Fig. 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 ± SE.

Fig. 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 normal differentiated murine podocytes, 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 ± SE. 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).
ultimately result in novel therapeutic interventions using P78-functional and structural integrity. Results of this study may P78-PEDF peptide has a direct effect in maintaining podocyte 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 in maintaining 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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