Exogenous kallikrein protects against diabetic nephropathy

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The kallikrein-kinin system has been shown to be involved in the development of diabetic nephropathy, but specific mechanisms are not fully understood. Here, we determined the renal-protective role of exogenous pancreatic kallikrein in diabetic mice and studied potential mechanisms in db/db type 2 diabetic and streptozotocin-induced type 1 diabetic mice. After the onset of diabetes, mice were treated with either pancreatic kallikrein (db/db + kallikrein, streptozotocin + kallikrein) or saline (db/db + saline, streptozotocin + saline) for 16 weeks, while another group of streptozotocin-induced diabetic mice received the same treatment after onset of albuminuria (streptozotocin + kallikrein, streptozotocin + saline). Db/m littersmates or wild type mice were used as non-diabetic controls. Pancreatic kallikrein had no effects on body weight, blood glucose and blood pressure, but significantly reduced albuminuria among all three groups. Pathological analysis showed that exogenous kallikrein decreased the thickness of the glomerular basement membrane, protected against the effacement of foot process, the loss of endothelial fenestrae, and prevented the loss of podocytes in diabetic mice. Renal fibrosis, inflammation and oxidative stress were reduced in kallikrein-treated mice compared to diabetic controls. The expression of kallikrein-kinin system.

KEYWORDS: diabetic nephropathy; fibrosis; inflammation; oxidative stress

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Diabetic nephropathy (DN), a major cause of end-stage renal disease, has become a worldwide public health issue.1,2 Several clinical studies provided convincing evidence that angiotensin-converting enzyme inhibitors, which inhibit angiotensin II formation and bradykinin degradation, had blood pressure-independent beneficial effects on DN in both types 1 and 2 diabetes.3,6 This highlighted that the kallikrein-kinin system (KKS) was also involved in DN. In an Akita diabetic mouse model lacking both types I and II bradykinin receptors (B1R and B2R, respectively), Kakoki et al.7 found that these mice displayed detrimental phenotypes, including urinary albumin excretion, glomerulosclerosis, and glomerular basement membrane thickening. In addition, overexpressing tissue kallikrein in mouse models ameliorated hypertension, insulin resistance, and DN.8-10 These studies suggested that KKS might be a useful target for the treatment of DN.

Tissue kallikrein is a serine protease that converts kini-nogen to the peptide hormone bradykinin and kallidin (in humans) or kallidin-like peptide (in rodents).11 Binding of bioactive bradykinin to B1R and/or B2R stimulates the production of nitric oxide (NO) and prostaglandins, which exerts protective effects on DN.12 Kwak et al.13 demonstrated that by implanting an osmotic bradykinin minipump, urinary albumin excretion in streptozotocin (STZ)-induced diabetic rats was reduced, and podocyte apoptosis was also amelio-rated. Overexpressing human tissue kallikrein has been shown to prevent the development of DN in rats.8,10 However, the mechanism through which kallikrein delayed the onset of DN was unclear.

To illuminate whether exogenous supplement of pancreatic kallikrein (PKK) could protect DN and to explore the mechanism, we used db/db mice, an obese type 2 diabetic
mouse, and STZ-induced type 1 diabetic mouse, as our models. We showed that PKK treatment not only significantly prevented the development of DN when given at the onset of diabetes, but also ameliorated DN when given after the onset of albuminuria. PKK improved renal pathologic alterations, which were accompanied by decreased renal fibrosis, inflammation, and oxidative stress product accumulation in both types of diabetic mice. Moreover, PKK significantly increased the expression of kininogen 1, tissue kallikrein, B1R, and B2R in diabetic mice. Our results suggest that the renal protective effects of PKK are mediated, in part, through the activation of KKS.

RESULTS

Animal characteristics

Data on body weight (BW), kidney weight (KW), blood glucose (BG), blood pressure (BP), and lipid profiles for db/db and db/m mice are summarized in Table 1. BG, BW, and serum triglyceride levels of db/db+saline (db/db+NS) and db/db+PKK groups were significantly higher than in db/m mice (P < 0.01), whereas the KW/BW ratio was lower than db/m mice (P < 0.01). However, there was no difference between db/db+NS and db/db+PKK groups. Other indexes including systolic and diastolic BP, total cholesterol, high- and low-density lipoprotein, showed no statistical differences among all three groups. The characteristics of STZ-induced diabetic mice was presented in Supplementary Table S1. Noteworthy, compared with NDC group, the KW/BW ratio was reversed in STZ+PKK group.

PKK decreased the urinary albumin/creatinine ratio in both types 1 and 2 diabetic mice

Albuminuria is considered as an important indicator of renal damage. Compared with db/m mice, db/db+NS mice showed a significantly increased urinary albumin to creatinine (Alb/Cre) ratio (Figure 1a), which was reduced by PKK. In STZ-induced diabetic mice, PKK showed the same effect (Figure 1b).

PKK protected the kidney structure in DN

Previous studies demonstrated that the increase in glomerular basement membrane (GBM) thickness was one of the most important pathological changes in DN. To verify whether PKK could protect the glomerulus of diabetic mice, we examined the ultrastructure of glomerulus using transmission electron microscopy (TEM). Our results showed that GBM thickness was significantly increased in the db/db+NS group compared with db/m mice, whereas PKK protected against this pathologic change (Figure 2a, red arrow). This was confirmed by semiquantitative analysis (Figure 2b). In addition, PKK also protected the endothelial fenestrae from fusion or loss (Figure 2c and d, yellow arrow), which partially explained the protective role of PKK against albuminuria. Similar results were observed in STZ-induced diabetic mice (Supplementary Figure S1).

Hematoxylin and eosin, periodic acid–Schiff (PAS), and Gomori trichrome reagent (Masson) staining were performed to examine the pathologic changes. Hematoxylin and eosin staining showed the inordinate arrangement of renal capillary in db/db+NS mice compared with db/m mice, whereas exogenous PKK prevented this pathologic disorder (Figure 2e). The percentages of PAS- or Masson-positive material, indicative of mesangial expansion and collagen deposition, respectively, and the glomerular sclerosis index were significantly increased in the diabetic glomeruli compared with db/m controls (Figure 2f–i). However, both were significantly attenuated in diabetic mice treated with PKK. Similar results for PAS and Masson staining were also observed in STZ-induced diabetic mice (Supplementary Figure S2).

PKK protected against the dysfunction and loss of podocytes in DN

To verify whether PKK protected podocytes from injury and dysfunction, we examined the ultrastructure of the foot process with TEM and Wilm's tumor (WT-1), a marker of a podocyte, with immunohistochemistry (IHC), respectively. Figure 3a shows that PKK protected against the effacement of the foot process in diabetic mice (Figure 3a and c; Figure 3b). We also showed that PKK increased the expression of keratin 18, a cadherin that promotes podocyte adhesion, in both types of diabetic mice.

Table 1 | Basic characteristics of mice

<table>
<thead>
<tr>
<th></th>
<th>db/m (n = 8)</th>
<th>db/db+NS (n = 8)</th>
<th>db/db+PKK (n = 8)</th>
<th>F value</th>
<th>P value</th>
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<tr>
<td>BW (g)</td>
<td>24.25 ± 3.06</td>
<td>42.64 ± 4.76*</td>
<td>42.64 ± 4.76a</td>
<td>38.82</td>
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<tr>
<td>BG (mM)</td>
<td>5.43 ± 0.64</td>
<td>27.37 ± 4.63*</td>
<td>26.90 ± 3.04a</td>
<td>86.35</td>
<td>&lt;0.0001</td>
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<td>SBP (mm Hg)</td>
<td>115.75 ± 8.25</td>
<td>110.20 ± 8.8</td>
<td>115.2 ± 8.76</td>
<td>0.99</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>86.25 ± 7.31</td>
<td>84 ± 5.4</td>
<td>84.4 ± 4.92</td>
<td>0.62</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>KW (mg)</td>
<td>307.8 ± 18.83</td>
<td>380.5 ± 13.52b</td>
<td>415.0 ± 15.44b</td>
<td>11.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>KW/BW (mg/g)</td>
<td>12.52 ± 2.27</td>
<td>9.02 ± 0.99*</td>
<td>9.83 ± 0.93b</td>
<td>7.27</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>TG (mM)</td>
<td>2.11 ± 0.3</td>
<td>4.06 ± 1.4*</td>
<td>3.68 ± 1.12c</td>
<td>6.88</td>
<td>&lt;0.01</td>
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<td>TCH (mM)</td>
<td>4.50 ± 0.63</td>
<td>5.31 ± 1.44</td>
<td>4.47 ± 0.75</td>
<td>1.82</td>
<td>&gt;0.05</td>
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<tr>
<td>HDL (mM)</td>
<td>2.19 ± 0.25</td>
<td>2.68 ± 0.71</td>
<td>1.99 ± 0.31</td>
<td>4.94</td>
<td>&gt;0.05</td>
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<tr>
<td>LDL (mM)</td>
<td>1.76 ± 0.62</td>
<td>1.53 ± 0.56</td>
<td>1.51 ± 0.57</td>
<td>0.45</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

BG, blood glucose; BW, body weight; DBP, diastolic blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; KW, kidney weight; KW/BW, kidney weight/ body weight; NS, saline; PKK, pancreatic kallikrein; SBP, systolic blood pressure; TCH, total cholesterol; TG, triglyceride.

Data are mean ± SEM.

*P < 0.001 versus db/m group.

aP < 0.01.

bP < 0.05.
Supplementary Figure S1, blue arrow). The number of WT-1–positive cells was significantly reduced in the db/db+NS group compared with the db/m group, whereas PKK treatment increased this number (Figure 3b and d).

Renal protective effects of PKK were associated with reduced fibrosis in DN
To verify whether PKK improved renal fibrosis, we examined renal fibrosis with IHC. Compared with the db/m group, db/db+NS mice showed significantly increased protein expression of fibronectin, collagen I, and TGF-β1 in both the glomeruli and interstitial area, which were reduced in db/db+PKK mice (Figure 4a–f). We also found that the mRNA expression of fibronectin and protein expression of collagen I was significantly decreased by PKK treatment (Figure 4h–i). Similar IHC results for collagen I and transforming growth factor (TGF)–β1 in STZ-induced diabetic mice are shown in Supplementary Figure S3.

Renal protective effects of PKK were associated with reduced inflammation and oxidative stress
CD68 is a marker of macrophages, which reflect inflammation. We found that CD68 expression was increased in db/db+NS mice compared with db/m mice. However, it was decreased in db/db+PKK mice (Figure 5a and c). In addition, the gene expression of interleukin-1β, an important cytokine in mediating renal inflammation, was elevated in db/db+NS mice compared with db/m mice. Notably, PKK also decreased the expression of interleukin-1β (Figure 5d). These results suggested that PKK exerted anti-inflammatory effects in diabetic kidneys.

Biomarkers including nitrotyrosine and glutathione (GSH) were detected to assess oxidative stress. Compared with db/m group, there was a significant accumulation of nitrotyrosine, a marker of oxidized protein, in the renal cortex of the db/db+NS group, which was reduced by PKK (Figure 5b and f). Furthermore, we found that PKK increased renal GSH, a major component of the antioxidative system, compared with saline (P < 0.05) (Figure 5e).

PKK activated the expression of the KKS in diabetic mice
The gene and protein expressions of renal KKS components are shown in Figure 6. Compared with db/m mice, db/db+NS mice showed a trend of increased mRNA levels of kininogen 1, kallikrein, B1R, and B2R, but this trend did not reach statistical significance (Figure 6a–d). However, the PKK treatment significantly increased mRNA expression. We also examined the protein expression of B2R in the kidneys of both types of diabetic mice (Figure 6i–k). Compared with db/m mice, the expression of B2R in the db/db+NS group was not statistically different, whereas PKK significantly increased B2R protein expression. In addition, the mRNA levels of these 4 genes and renal bradykinin concentration were all increased in the STZ+PKK group compared with the STZ+NS group (Figure 6e–h; Supplementary Figure S4).

PKK treatment reduced the Alb/Cre ratio and serum creatinine levels when given after the onset of albuminuria
In order to test whether PKK is capable of reversing DN, we treated the STZ-induced diabetic mice with PKK for 8 weeks after the onset of albuminuria. Data on body weight (BW), KW, BG, and BP for these mice are summarized in Supplementary Table S2. As shown in Figure 7a, after 8 weeks of diabetes, the Alb/Cre ratio was significantly increased in STZ+NS mice compared with nondiabetic control (NDC) mice (the prime symbols indicate that these mice are relevant to the period before the PKK or saline treatment). However, after another 8 weeks of PKK treatment, the increased Alb/Cre ratio was reversed. Moreover, the serum creatinine level was also decreased in the STZ+PKK group compared with the STZ+NS group (Figure 7b).

PKK protected the kidney structure when given after the onset of albuminuria in STZ-induced diabetic mice
As shown, GBM thickness was significantly increased in the STZ+NS group compared with NDC mice, whereas PKK ameliorated this pathologic change (Figure 8a, red arrow). In addition, PKK also improved the fusion or loss of endothelial fenestrae (Figure 8a, yellow arrow) and reversed
the effacement of the foot process in diabetic mice compared with STZ
0þ NS mice (Figure 8a, blue arrow). Semiquantitative analysis further confirmed our findings (Figure 8b–d). The quantitative thickness of the GBM and endothelial fenestrations for these 3 groups. Red and yellow arrows point to the GBM and fenestrae of endothelial cells, respectively. (e,f,h) Morphometric analysis of the PAS- or Masson-positive area was performed in renal sections of all mice. Representative images of kidney tissue stained with HE, PAS, and Masson for the db/m, db/db+NS, and db/db+PKK groups are presented (original magnification ×40). (g,i) The glomerular sclerosis indexes for PAS and Masson staining, respectively. Data shown are mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 versus the db/m group; #P < 0.05, ###P < 0.001 versus the db/db+NS group. GBM, glomerular basement membrane; GBMT, glomerular basement membrane thickness; HE, hematoxylin and eosin; NS, saline; PAS, periodic acid–Schiff; PKK, pancreatic kallikrein.

PKK restored podocyte number, reduced renal fibrosis and inflammation, and increased urinary NO level when given after the onset of albuminuria

Further, we performed WT-1, collagen I, TGF-β1, and CD68 IHC staining, which showed that PKK restored the number of podocytes (Figure 9a and b), which was consistent with the TEM results. The antifibrotic effects of PKK, which were verified by collagen I IHC staining (Figure 9c and d), were accompanied by decreased TGF-β1 and CD68 protein expression (Figure 9e–h). In addition, we tested the urinary nitrate levels, which reflected the NO levels. Although without reaching statistical significance, the NO level tended to be decreased in STZ+NS mice compared with NDC’ mice (Supplementary Figure S5) (P = 0.0874), whereas this tendency was partially reversed by PKK (P = 0.0776). We also tested the expression of renal KKS by detecting the mRNA expression of kininogen 1, kallikrein, B1R, and B2R (Figure 9i–l). Similarly, PKK treatment significantly increased mRNA expression on these 4 genes compared with the other 2 groups, indicating that KKS activation might partially mediate the protective effect of PKK on DN.
DISCUSSION

The KKS was recognized at the discovery that urine contains a vasodilator substance further identified in pancreatic extracts and named kallikrein in 1930.\textsuperscript{16,17} It is now well established that the KKS is a complex enzymatic system and expressed ubiquitously in many organs, including kininogen, serum or tissue kallikreins and other serine proteases, kinins and their derivatives (Supplementary Figure S6).\textsuperscript{4,18} Emerging evidence suggests a protective role for the KKS in the pathogenesis of DN. B2R knockout Akita mice and STZ-induced tissue kallikrein knockout diabetic mice displayed more severe albuminuria and glomerulosclerosis.\textsuperscript{7,19} Moreover, previous studies demonstrated that bradykinin, the major mediator of the KKS, inhibited high glucose- and growth factor–induced collagen synthesis in mesangial cells of diabetic mice.\textsuperscript{20} Exogenous supply of KKS activators such as kinin, human tissue kallikrein cDNA (rAAV-HK), and B2R agonist effectively improved DN in diabetic models.\textsuperscript{8,21,22}

In the current study, we treated type 1 and 2 diabetic mice with PKK and found that it not only protected against the development of DN, but also ameliorated DN when given after the onset of albuminuria. Our results demonstrated that exogenous PKK decreased the Alb/Cre ratio, ameliorated renal pathologic changes including a thickened GBM, effacement of foot processes, and loss of endothelial fenestrae. We also evaluated the fibrosis, inflammation, and oxidative stress as they are the most important pathways in the development of DN.\textsuperscript{23–28} We found that exogenous PKK reduced...
and reversed these pathologic pathways, suggesting that PKK has renal protective effects in diabetic mice.

After confirming the renal protective effect of exogenous PKK, we explored the underlying mechanism. Because the increased substrate (kininogen) or enzyme (tissue kallikrein) could result in producing more bradykinin and NO, which were the major mediators of KKS, we examined the expression of every component of KKS in our animals. We found that PKK treatment increased the gene expression of tissue kininogen 1, kallikrein 1, B1R and B2R and protein expression of B2R. Moreover, we found that PKK could increase the renal bradykinin level in STZ-induced diabetic mice, which may partially explain the protective role of PKK in DN.

Previous studies demonstrated that the gene expression of KKS, including tissue kallikrein, kininogen, B1R, and B2R, was increased in the kidney of 3-month-old C57Bl/6J db/db mice. However, in our mouse models, gene expression of KKS in the kidney was not statistically different from that in NDC mice. This discrepancy may be due, at least in part, to differences in genetic background, severity of hyperglycemia, and duration of diabetes. Interestingly, our results suggest a “positive feedback” mechanism in the KKS in our mouse models. In diabetic mice treated with PKK, the expression of both B1R and B2R were significantly increased, which implies the upregulation of both receptors by the elevated bradykinin level. Previous studies demonstrated that the main regulatory mechanism of bradykinin receptor expression was at the transcriptional and posttranscriptional levels. In trauma, acute inflammation, and stress, upregulation of B1R and B2R occurred under the control of some cytokines, including interleukin-1β, TNF-α, and lipopolysaccharide. Previous studies by Saifudeen et al. suggested an important role of p53 in upregulating the G protein–coupled receptor B2R during kidney development in mice. Specifically, p53-induced B2R activation was mediated by the direct binding to the promoter region of the BK2 gene. Furthermore, the combined interactions among transcriptional factors or coactivators including p53, CREB, KLF-4, and CBP.
appeared to be critical in the regulation of B2R gene expression, particularly during terminal nephron differentiation.\textsuperscript{35} In cultured rat aortic vascular smooth muscle cells, Yan et al.\textsuperscript{36} found that bradykinin could upregulate the expression of B2R through mitogen-activated protein kinase and phosphatidylinositol-3' kinase/Akt signaling pathways.

Figure 5 | Renal protective effects of PKK were associated with reduced inflammation and oxidative stress in diabetic kidneys. (a,b) Representative IHC micrographs of kidney tissue stained with CD68 and nitrotyrosine for the db/m, db/db+NS, and db/db+PKK groups, respectively. Original magnification ×40. (c) The IHC scores of kidney sections for CD68 IHC staining. (d) The gene expression of IL-1β for these 3 groups. (e) The IHC scores of kidney sections for nitrotyrosine IHC staining. (f) GSH level for these 3 groups. Data shown are mean ± SEM. \( ^* P < 0.05, ^{**} P < 0.01 \) versus the db/m group; \( ^{##} P < 0.05, ^{###} P < 0.01 \) versus the db/db+NS group. GSH, glomerular stimulating hormone; IHC, immunohistochemistry; IL-1β, interleukin-1β; NS, saline; PKK, pancreatic kallikrein.

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found that B1R and B2R gene expression was increased by diabetes in both WT and tissue kallikrein-null mice, so they ruled out the possibility of ligand-induced receptor upregulation. However, they did not explore the potential mechanism further. In our mouse model, the gene expression of B1R and B2R was not significantly increased in diabetic mice compared with NDC mice. Nevertheless, exogenous PKK significantly increased gene expression of both receptors, which indicated that this upregulation might be mediated by ligand-induced effects.

Interestingly, a recent paper demonstrated that kallistatin, which could inhibit endogenous tissue kallikrein activity, had a renoprotective effect on DN. The discrepancy between this research and ours may be due to different
models. In the study by Yiu et al., kallistatin overexpression was genetically induced specifically in renal tubules by ultrasound microbubble–mediated gene transfer, whereas in our study, PKK was systemically administered, which might lead to broader effects. Yiu et al. concluded that the renoprotective effect of kallistatin was due to the reduced oxidative stress and systolic blood pressure. However, in our models, PKK decreased oxidative stress, but had no effects on blood pressure. Moreover, previous studies showed that tissue kallikrein improved insulin resistance and metabolic profiles, which might contribute the renal protection. In our study, PKK-treated mice showed a decreasing trend of plasma total cholesterol and triglycerides, which might partially mediate the protective effects.

Our findings showed that PKK treatment improved DN in diabetic mice through the regulation of KKS, mediated at least in part by activation of B1R and B2R. Further studies are warranted to examine the mechanism of a protective role of PKK in DN.

In summary, we found that exogenous PKK has renal protective effects in type 1 and 2 diabetic mice, exemplified by the improvement of pathologic structures, reduced proteinuria, glomerulosclerosis, renal inflammation, and oxidative stress. Thus, exogenous PKK could be an effective medication to protect against DN.

**MATERIALS AND METHODS**

**Animal study**

All animal studies were performed in accordance with approved Institutional Animal Care and Use Committee protocols at Fudan University. Sixteen male C57BLKS/J db/db mice (obtained from the Model Animal Research Center, MARC, Nanjing University, Nanjing, China) weighing 22 to 26 g were injected either with saline \( n = 8 \), 0.1 ml/10 g, db/db+NS group) or PKK intraperitoneally (Qianhong Bio-pharma Company, Changzhou, China) \( n = 8 \), 60 U/kg, db/db+PKK group) for 16 weeks. In addition, littermate db/m mice \( n = 8 \), db/m group) were given saline (0.1 ml/10 g) as the NDC group. For the type 1 diabetic mouse model, 5-week-old male DBA mice (obtained from Shanghai Laboratory Animal Center, Shanghai, China) were made diabetic as described previously. Brieﬂy, mice were injected with STZ (50 mg/kg, i.p.) on 5 consecutive days. After the onset of diabetes, determined by hyperglycemia as BG reached 16.7 mmol/l at 1 week post-injection, mice were separated into either a PKK-treated group \( n = 8 \) (STZ+PKK group) or a control group treated with saline \( n = 8 \) (STZ+NS group). After 12 weeks of treatment, the mice were killed. For another group of STZ-induced mice \( n = 12 \), designed as the STZ+NS group), we measured the Alb/Cre ratio at 8 weeks after the onset of diabetes and significant albuminuria had developed compared with the corresponding control NDC group. Then these mice received either PKK \( n = 6 \), designed as the STZ+PKK group) or saline \( n = 6 \), designed as the STZ+NS group) for another 8 weeks. The NDC group \( n = 6 \) received saline. Mice were housed in a temperature-controlled room and given free access to water and standard laboratory chow during the whole study period.

BW and BG were checked weekly, and KW was measured when the mice were killed. BP was measured monthly by tail-cuff plethysmography (BP-98A, Softron Beijing Incorporated, Beijing, China). Mice were housed in metabolic cages, and 24-hour urine samples were collected. The urinary albumin level was measured by enzyme-linked immunosorbent assay (Bethyl Laboratory Inc., Houston, TX, USA). Urinary creatinine concentration was determined using a chemiluminescence assay (Exocell, Philadelphia, PA, USA), and Alb/Cre ratio was calculated.

**Histologic evaluation**

Partial cortices of kidneys were fixed with 4% paraformaldehyde overnight. Tissues were embedded in paraffin, stained with hematoxylin and eosin or PAS or with Masson reagent, and examined under an optical microscope. The glomerular sclerosis index was calculated as previously described. Renal tissue was also examined with TEM after fixation with 10% glutaraldehyde. The GBM thicknesses in TEM pictures were measured by ImageJ software. The method to measure the width of the foot process was previously described in detail. Foot process effacement is determined as the length of the effacement divided by the length of
Figure 8 | PKK protected the kidney structure when given after the onset of albuminuria in STZ-induced diabetic mice. (a) The representative electronic micrographs for the glomerular filtration barrier in STZ-induced diabetic mice that were treated after the (Continued)
the capillary. Five glomeruli per mouse and 5 open random capillary loops per glomerulus for 3 mice in each experimental group were also measured by using ImageJ. As to the quantification of endothelial fenestrations, values are expressed as mean number of endothelial fenestrations per 100 μm of capillary loop perimeter.43

Figure 9 | PKK restored podocyte number, reduced renal fibrosis and inflammation, and activated the expression of the kallikrein-kinin system when given after the onset of albuminuria. (a,c,e,g) Representative immunohistochemical micrographs of kidney tissue stained with WT-1, collagen I, TGF-β1, and CD68 for NDC’, STZ+NS’, and STZ+PKK’ groups. Original magnification ×40. (b,d,f,h) The immunohistochemical scores for WT-1, collagen I, TGF-β1, and CD68 IHC staining, respectively. (i–l) The RQ values of kininogen1, kallikrein 1, B1R, and B2R. Data shown are mean ± SEM. \( ^{\dagger}p < 0.05, ^{\ddagger}p < 0.01, ^{\ddagger\ddagger}p < 0.001 \) vs. the NDC’ group, \( ^{\dagger\dagger}p < 0.01, ^{\ddagger\ddagger}p < 0.001 \) vs. the STZ’ + NS group. B1R, bradykinin receptor type I; B2R, bradykinin receptor type II; NDC, nondiabetic control; NS, saline; PKK, pancreatic kallikrein; RQ, relative quantity; STZ, streptozotocin; TGF-β1, transforming growth factor β1; WT-1, Wilms tumor. STZ’’ and NDC’’ refer to the pre-PKK or saline treatment period.
Table 2 | Sequences of primers used for real-time PCR

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<td>IL-1β</td>
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<td>Fibronectin</td>
<td>5'-TCCCGGGCGAAAGATCATT-3'</td>
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<td>Kininogen 1</td>
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<td>B1R</td>
<td>5'-GCCAGGTGGTGTACATCCTG-3'</td>
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<td>B2R</td>
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<tr>
<td>β-Actin</td>
<td>5'-GAGACCTTCAACCCACCCGC-3'</td>
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ACE, angiotensin-converting enzyme; B1R, type I bradykinin receptor; B2R, type II bradykinin receptor; IL-1β, interleukin-1β; PCR, polymerase chain reaction.

Immunohistochemistry
Expression of WT-1 (sc-192, Santa Cruz Biotechnology Inc., Dallas, TX, USA), TGF-β1 (sc-146, Santa Cruz Biotechnology Inc.), fibronectin (ab2413, Abcam, Cambridge, MA, USA), collagen I (ab34710, Abcam), CD68 (MCA1957-GA, AbD Serotec Company, AbD Serotec, Kidlington, UK), and nitrotyrosine (2459610, Millipore, Billerica, MA, USA) was examined on paraffin-embedded renal tissue sections (5 mm) by IHC analysis, as described previously. The dilution was 1:100, 1:100, 1:100, 1:200, 1:100, and 1:200, respectively. Analysis of WT-1 and CD68 staining and the number of positively stained cells per glomerular cross-section area in the kidney sections was performed, as described previously. For other antibodies, each section was captured for at least 10 images, and all these images were quantified by Image Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA). Data were expressed as the ratio of integrated optical density to area.

RNA extraction and real-time polymerase chain reaction
Kidneys from control and diabetic mice were collected after killing, and cortices were isolated to extract RNA using Trizol (Invitrogen, Carlsbad, CA, USA) followed by the manufacturer’s instructions. Total RNA was dissolved in the ethylenediamine tetraacetic acid–free water. The RNA concentration was determined with a spectrophotometric method according to the manufacturer instruction of the spectrophotometer (Nanodrop 2000c, Thermo Fisher Scientific, Waltham, MA, USA) by absorbance at 260 nm. According to the manufacturer’s protocols, RNA (2 μg) was converted to cDNA (G490, Abm Canada, Milton, Ontario, Canada) and then reverse transcriptase–polymerase chain reaction was conducted on ABI 7500 (Applied Biosystems, Foster City, CA, USA) with SYBR Green PCR Master Mix (Applied Biosystems). The primers used in this paper are shown in Table 2. Each sample was run in triplicate to permit precise quantification of the gene normalized to the β-actin gene.

Measurement of renal GSH, renal bradykinin, and urinary nitrate levels
Renal GSH, bradykinin, and nitrate levels were determined with a spectrophotometric method according to the manufacturer’s instructions (703002, Cayman Chemical Companies, Ann Arbor, MI, USA; EK-009-01, Phoenix Pharmaceuticals, Burlingame, CA, USA; 780001, Cayman Chemicals). The results for GSH and bradykinin were standardized to homogenate protein levels.

Protein extraction and Western blot analysis
The renal cortex of the kidney was homogenized in an ice-cold lysis buffer containing 150 mmol/l NaCl, 50 mmol/l Tris-HCl (pH 8.0), 0.1% sodium dodecylsulfate, 1% Nonidet P-40, and protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany). These samples were resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were incubated with rabbit anti-collagen I antibody (1:5000), rabbit anti-B2R antibody (1:2000), and rabbit anti–β-actin antibody (1:5000), washed and incubated with goat anti-rabbit peroxidase–coupled secondary antibodies (1:10,000). The blots were visualized by an enhanced chemiluminescence detection system (PerkinElmer, Boston, MA, USA).

Statistical analysis
All data were expressed as mean ± SEM from 3 independent experiments. Statistical analysis was performed using the statistical package SPSS for Mac Version 20.0 (SPSS, Inc., Chicago, IL, USA). The significance of the differences in mean values among different groups was evaluated using 1-way analysis of variance followed by the Tukey test. P values <0.05 were considered to be statistically significant.

DISCLOSURE
All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL
Figure S1. PKK protected the ultrastructure of glomerular filtration barrier in STZ-induced diabetic mice. (A) The representative electronic micrographs of glomerular filtration barrier in STZ-induced diabetic mice. (B–D) The quantitative thickness of GBM, foot process, and endothelial fenestrations for these three groups. Red, blue, and yellow arrows point to GBM, foot process, and fenestrae of endothelial cells, respectively. Data are means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 versus NDC group; †P < 0.01, ††P < 0.001 versus STZ+NS group.

Figure S2. PKK reduced glomerular mesangial expansion and collagen deposition in STZ-induced diabetic mice. (A,C) Representative images of PAS and Masson staining for NDC, STZ+NS, and STZ+PKK groups (original magnification ×40). (B) The glomerular sclerosis indexes for PAS and Masson staining, respectively. Data are means ± SEM. **P < 0.01 versus NDC group; ††P < 0.01 versus STZ+NS group.

Figure S3. Renal protective effects of PKK were associated with reduced fibrosis and inflammation in STZ-induced diabetic kidneys. (A,C,E) Representative immunohistochemical micrographs pictures of kidney tissue stained with collagen I, TGF-β1, and CD68 for NDC, STZ+NS, and STZ+PKK groups. Original magnification ×40. (B,D,F) The immunohistochemical scores for these 3 IHC staining
respectively. Data are means ± SEM. $^{\text{p}} < 0.01$, $^{\text{pp}} < 0.001$ versus NDC group; $^{\text{ppp}} < 0.001$ versus STZ + N5 group.

**Figure S4.** PKK treatment tended to increase the renal bradykinin level in STZ-induced diabetic mice. The renal bradykinin level in NDC, STZ + N5, and STZ + PKK groups. Data are means ± SEM. $^p < 0.05$ versus NDC group; $^{pp} < 0.05$ versus STZ + N5 group.

**Figure S5.** PKK likely increased urinary nitrate levels in diabetic mice. The urinary nitrate level in NDC, STZ + N5, and STZ + PKK groups. Data are means ± SEM.

**Figure S6.** Renal kallikrein-kinin system (KKS) and renin-angiotensin-aldosterone system (RAAS). The components and molecular interaction between renal kallikrein-kinin system and renin-angiotensin-aldosterone system. Bradykinin, kallikardin (humans), and kallidin-like peptide (rodents) are generated from kinogenins by kallikreins. By activating bradykinin receptors (type 1 and type 2, B1R and B2R), all kinins could stimulate two intracellular signaling pathways, phospholipase A2 (PLA2)-dependent and phosphatidylinositol-specific phospholipase C (PI-PLC)-dependent pathways. This activation could result in the production of nitric oxide (NO) and prostaglandins (PGs), which may further protect the renal protective roles in DN. Kallikrein could also convert pro-renin into renin, which further catalyze angiotensinogen into angiotensin I. Importantly, kinins could be degraded into inactive forms by kininase II (ACE), while angiotensin I could be transformed into angiotensin II. LMWK, low molecular weight kininogen; HMWK, high molecular weight kininogen; Gg, Gq protein; Gi, Gi protein; iNOS, inducible nitric oxide synthase; eNOS, endothelial nitric oxide synthase; ER, endoplasmic reticulum; PRCP, lysosomal pro-X carboxypeptidase; AT1R, type 1 angiotensin receptor; AT2R, type 2 angiotensin receptor; AT1(R)-2, angiotensin receptor for angiotensin II (1-7). Adapted with permission from Tomita H, Sanford RB, Smithies O, et al. The kallikrein-kinin system in diabetic nephropathy. *Kidney Int*. 2012;81:733–744.

**Table S1.** Basic characteristics of DBA mice treated for 12 weeks.

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<th>Table S2. Basic characteristics of mice.</th>
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<td>Supplementary material is linked to the online version of the paper at <a href="http://www.kidney-international.org">www.kidney-international.org</a>.</td>
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**REFERENCES**


