

# Inhibition of inflammation by pentosan polysulfate impedes the development and progression of severe diabetic nephropathy in aging C57B6 mice

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Inflammation has a key role in diabetic nephropathy (DN) progression. Pentosan polysulfate (PPS) has been shown to decrease interstitial inflammation and glomerulosclerosis in 5/6 nephrectomized rats. Since PPS has an excellent long-term safety profile in interstitial cystitis treatment, and we recently found that old diabetic C57B6 mice develop DN characterized by extensive tubulointerstitial inflammatory lesions that mimics human DN, we examined the effect of PPS on old diabetic mice. We also examined the anti-inflammatory properties of PPS in renal cells *in vitro*. Diabetes was induced with streptozotocin in 18 months female (early aging) C57B6 mice. Mice were then randomized to receive oral PPS (25 mg/kg/day) or water for 4 months. The effect of PPS on NF- $\kappa$ B activation and on TNF $\alpha$ , high glucose or advanced glycation end products (AGEs) stimulated proinflammatory gene expression in renal cells was examined. We found that PPS treatment preserved renal function, significantly reduced albuminuria, and markedly decreased the severity of renal lesions, including tubulointerstitial inflammation. PPS also reduced upregulation of TNF $\alpha$  and proinflammatory genes in aging diabetic kidneys. Furthermore, PPS suppressed NF- $\kappa$ B, decreased the proinflammatory actions of TNF $\alpha$ , and decreased high glucose and AGEs stimulated MCP-1 production *in vitro*. Finally, PPS decreased TNF $\alpha$ -induced increase in albumin permeability in podocyte monolayers. In conclusion, PPS treatment largely prevents the development/progression of nephropathy in aging diabetic mice. As this may be mediated by suppression of TNF $\alpha$ , high glucose, and AGE-stimulated NF- $\kappa$ B activation and inflammation *in vitro*, the *in vivo* blockade of DN may be due to the anti-inflammatory properties of PPS.

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**KEYWORDS:** aging; diabetic nephropathy; inflammation; PPS; TNF $\alpha$

Diabetic nephropathy (DN) is the leading cause of end-stage renal disease.<sup>1</sup> Currently, one of the standards of care for DN patients includes blockade of angiotensin II by angiotensin-converting enzyme inhibitor (ACEI) and/or angiotensin receptor blocker (ARB). Both ACEI and ARB have been shown to slow the progression of DN to some degree.<sup>2–4</sup> As most patients continue to progress to renal failure,<sup>5</sup> new therapeutic agents are urgently needed.

Multiple pathways, including dysregulation of renal vasculature, activation of protein kinase C, increased transforming growth factor  $\beta$ , accumulation of advanced glycation end

products (AGEs) and excessive oxidative stress have all been implicated in the pathogenesis of DN.<sup>6–9</sup> Growing evidence also supports an important role for chronic inflammation in DN progression.<sup>10,11</sup> For instance, inflammation is critically involved in tubulointerstitial lesions, a key component in renal function decline.

Although the detailed inflammatory signaling pathway(s) involved in the pathogenesis of DN are largely unclear, several anti-inflammatory drugs, such as the inhibitor(s) of cyclooxygenase 2 and rapamycin have been tested either experimentally or clinically for the management of DN.<sup>12,13</sup>

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Pentosan polysulfate (PPS) is a mixture of semisynthetic sulfated polyanions that is approved by the FDA as an only oral medication for the treatment of interstitial cystitis, an inflammatory-like disease.<sup>14</sup> The mechanism of anti-inflammatory action of PPS is not clear. It is reported that PPS decreases lipopolysaccharide-mediated NF $\kappa$ B activation, suppresses leukocyte elastase and inhibits complement activity.<sup>15–17</sup> As PPS has been found to substantially decrease tubulointerstitial inflammation and preserve renal function in 5/6 nephrectomized rats,<sup>18</sup> we asked if the anti-inflammatory effect of PPS reduced DN in aging mice, which develop severe nephropathy, including typical tubulointerstitial inflammatory lesions. We also tested the effect of PPS on high glucose, AGEs and TNF- $\alpha$ -stimulated inflammation in renal cells *in vitro*.

## SUBJECTS AND METHODS

### Animals

Seventeen-month-old female C57B6 mice were obtained from National Institute on Aging and were injected with low dose of streptozotocin (50  $\mu$ g/g) every 3 days for a total of 5–8 injection to induce diabetes (<http://www.diacomp.org/shared/protocols.aspx?model=5>).<sup>19</sup> Mice with stable hyperglycemia ( $\geq 250$  mg/dl) at 18 months of age were selected for the study. Eighteen-month-old diabetic mice were randomly divided into PPS-treated ( $n = 10$ , PPS 25 mg/kg/day in the drinking water) and control ( $n = 11$ ) groups. This dosage of PPS was selected based on previous reports.<sup>18</sup> The pH of drinking water with or without PPS remains at 7. Diabetic mice were followed for 4 months without insulin treatment. Mouse was housed individually and water intake was recorded every other day. Body weight and blood glucose levels were monitored weekly. Additionally, urine ketones were examined. Urine albumin excretion was measured bi-weekly as previously described.<sup>20</sup> Urine creatinine levels were measured in the same samples and the urine albumin excretion rate was expressed as the ratio of albumin to creatinine. Serum creatinine levels were examined at killing. High-performance liquid chromatography was applied for determining serum creatinine levels using the method described by Yuen *et al.*<sup>21</sup>

### Renal Histology and Morphometry

Mice were killed 4 months after diabetes. The kidneys were perfused with PBS solution and subsequently one kidney was perfusion-fixed *in situ* with 4% paraformaldehyde.<sup>20</sup> The tissues were embedded in glycol methacrylate or low melting paraffin, and stained with periodic acid Schiff (PAS) and Masson's Trichrome. Tissues were also post-fixed for 1 h in 1.0% osmium tetroxide, prestained in 1.25% uranyl acetate for 1 h, dehydrated through a series of graded alcohol solutions and embedded in EPON epoxy resin for electron microscopy. The glomerular volume and mesangial area were determined by examining PAS sections using a digitizing tablet and video camera.<sup>19,20</sup> The relative mesangial area was

expressed as mesangial/glomerular surface area. Glomerular cell number was determined by counting the nuclear number in at least 30 glomeruli of each section. The glomerular basement membrane thickness was measured by the orthogonal intercept method on electron microscopic images. To determine the number of glomerular fenestrae, the length of fenestrated or unfenestrated glomerular capillary endothelial cytoplasm was measured. Fenestrated endothelial cell cytoplasm was generally thin whereas the thickness of the unfenestrated endothelial cell cytoplasm was twice or more increased.<sup>22</sup> Tubulointerstitial lesions were scored from 0 to 4 (0, no changes; 1+, changes affecting <25% of the area; 2+, changes affecting 25–50% of the area; 3+, changes affecting 50–75% of the area; 4+, changes affecting 75–100% of the area) based on tubular atrophy, increase in the thickness of basement membranes and interstitial area, and clusters of inflammatory cells.<sup>23</sup>

### Immunohistochemistry

Paraffin sections from diabetic and PPS-treated mice were deparaffinized before staining for inflammation makers, such as macrophages (F4/80, Caltag Laboratories, Burlingame, CA, USA) and phosphorylated NF- $\kappa$ B (p65 (Ser276), Cell Signaling Technology, Inc., Danvers, MA, USA). Additionally, staining for albumin was performed (rabbit anti-mouse albumin, Bethyl Labs, Montgomery, TX, USA). In brief, sections were soaked three times in xylene and passed serially through decreasing concentrations of alcohol. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide. Antigen unmasking was performed either by putting sections in 0.1% Triton X-100 for 30 min (for F4/80 staining) or heating sections in citrate buffer for 15 min in a microwave oven (for phosphorylated NF- $\kappa$ B staining). After incubating with primary antibody at 4 °C overnight, sections were reacted with biotin-labeled secondary antibody in the Vector VECTASTAIN Elite ABC Kit (Vector Laboratories). Positive staining was revealed with Vector DAB Substrate Kit (SK-4100, Vector Laboratories). Nuclei were counter-stained with hematoxylin in some of sections (Millipore, Billerica, MA, USA). Stained sections were examined under light microscope (Zeiss Axioskop, Germany). The area in renal cortex was digitized under  $\times 10$  objective low power with a Sony 3CCD color video camera and a meta imaging series software (Molecular Devices, Downingtown, PA, USA).<sup>24</sup> Positively stained area of monocyte/macrophages was measured and expressed as the percentage of total cortical area.<sup>24</sup> The intensity of the staining of albumin in tubules was assessed using the color differentiation program. NF- $\kappa$ B-positive cells were counted under microscope ( $\times 40$  objective).

### Proinflammatory Gene mRNA Levels in Aging Diabetic Kidneys

Total RNA was isolated from renal cortex using a PureYield RNA Midiprep kit (Promega, Madison, WI, USA). The preparation was free of DNA contamination and 500 ng

of total RNA from each sample was reverse-transcribed as previously described.<sup>19,20</sup> The levels of MCP-1 (monocyte chemoattractant protein-1), CXCL1 (c-x-c motif ligand 1), RANTES (regulated on activation, normal T-cell expressed and secreted) and IL-6 (interleukin 6) mRNA were determined by real-time polymerase chain reaction used the primers as previously described.<sup>24</sup> mRNA levels were corrected by the levels of  $\beta$ -actin or GAPDH mRNA in the same sample. The expression of TNF $\alpha$  mRNA in kidney was determined by both a real-time and a regular PCR using the primer of forward, 5'-GCGACGTGGAAGTGGCAGAAG-3', reverse, 5'-GGTACAACCCATCGGCTGGCA-3'. GAPDH and  $\beta$ -actin mRNA levels were determined at the same time.

### Effect of PPS on TNF $\alpha$ -Stimulated Upregulation of Proinflammatory Genes in a Proximal Tubular Cell Line

As TNF $\alpha$  has an important role in renal inflammation and proximal tubular cells are important target for TNF $\alpha$  action, we examined the effect of PPS on TNF $\alpha$ -stimulated inflammation in proximal tubular cells under normal glucose condition. A proximal tubular cell line obtained from mice transgenic for SV40 T antigen was kindly provided by Dr. Neilson.<sup>25</sup> In all,  $1 \times 10^5$  cells were seeded in each well of a six-well plate. After placing the cells in 0.1% FBS medium for 24 h, cells were pretreated with PPS (200  $\mu$ g/ml) for 1 h before the addition of TNF $\alpha$  (10 ng/ml). This dosage of PPS was selected based on the calculation that a likely drug concentration achieved from oral absorption of 6% of given 100 mg/kg of PPS in rats.<sup>18</sup> Total RNA was collected from cells 4 h after the treatment. MCP-1, RANTES, CXCL1, MIP-2 (macrophage inflammatory protein 2), ICAM-1 (intracellular adhesion molecule 1) and iNOS (inducible nitric oxide synthase) mRNA levels were determined 4 h later by real-time PCR.  $\beta$ -Actin mRNA levels served as an internal control. The primers used for the measurement were MIP2, forward, 5'-TCCAGAGCTTGAGTGTGACG-3'; reverse, 5'-TTCAGGGTCAAGGCAAACCTT-3'; ICAM-1, forward, 5'-TGC TGCAGATGCTGTGAGAGT-3'; reverse, 5'-AAACCCTCGAC CCATGTGATC-3'; and iNOS, forward, 5'-GAAGAAAACCC CTTGTGCTG-3'; reverse, 5'-CTGTGCTGTCCAGTGAGG-3'. Some of cells were treated with TNF $\alpha$  in the presence or absence of PPS for 24 h. The amount of MCP-1 or RANTES production by the cells was measured by ELISA with kits from Invitrogen Corporation (Carlsbad, CA, USA).

### Effect of PPS on TNF $\alpha$ -Stimulated NF- $\kappa$ B Activation in a Proximal Tubular Cell Line

The activation of NF- $\kappa$ B and mitogen-activated protein kinases including p38, JNK (Jun N-terminal kinase) and ERK (extracellular signal-regulated kinase) pathways have an essential role in proinflammatory actions of TNF $\alpha$ . Thus, we examined the effect of PPS on the phosphorylation of I $\kappa$ B, ERK, p38 and JNK by TNF $\alpha$ . Proximal tubular cells were allowed to grow to 70% confluency in 60 mm<sup>2</sup> Petri-dish. Cell lysates were collected before or 15, 30, 60 and 120 min

after TNF $\alpha$  treatment (10 ng/ml). Cells in some dishes were pre-incubated with PPS (400  $\mu$ g/ml) for 1/2 h before exposure to TNF $\alpha$ . The levels of phosphorylated I $\kappa$ B, ERK, p38 and JNK were determined by western blots. Briefly, samples containing equal amounts of protein (10–20  $\mu$ g per lane) were loaded onto SDS-PAGE gels. After electrophoresis, proteins were transferred to PVDF membranes and blotted with antibody against phosphorylated I $\kappa$ B, ERK, p38 or JNK (Cell Signaling, Boston, MA). After the first analysis, the membranes were stripped to re-probe with antibody against total I $\kappa$ B, ERK, p38 or JNK, and finally with antibody against  $\beta$ -actin (Cell Signaling).

To further determine the effect of PPS on NF- $\kappa$ B transcription activity, proximal tubular cells were transfected with a NF- $\kappa$ B reporter in the presence or absence of PPS (50–200  $\mu$ g/ml) and TNF $\alpha$  (10 ng/ml). Cells were also transfected with a cDNA expression vector containing constitutively active I $\kappa$ B, dominant-negative IKK or IKK. A  $\beta$ -galactosidase cDNA expression vector was co-transfected with the NF- $\kappa$ B reporter to serve as an internal control for transfection efficiency. Luciferase and  $\beta$ -galactosidase activity were measured using substrate assays as previously described.<sup>26</sup> The same amount of DNA (1  $\mu$ g per well of 24-well plates) was used for each transfection throughout the experiments.

Additionally, NF- $\kappa$ B DNA binding was examined by electrophoretic mobility shift assay (EMSA), as previously described.<sup>27</sup> Briefly, proximal tubular cells were pre-incubated with or without PPS (800  $\mu$ g/ml) for 1/2 h before the addition of TNF $\alpha$  (10 ng/ml). Nuclear protein was extracted from cells 1 h after treatment. A NF- $\kappa$ B probe (5'-AGTTG AGGGGACTTTCCAGGC-3') was prepared by annealing complementary single-stranded oligonucleotides with 5'-AC TG-3' overhangs (MWG Biotechnologies, Inc.) and was labeled by filling in with [<sup>32</sup>P]dGTP and [<sup>32</sup>P]dCTP using Klenow enzyme. Labeled probes were purified with NuTrap purification columns (Roche Applied Science). EMSAs were performed using 10<sup>5</sup> c.p.m. of labeled probe and 10  $\mu$ g of nuclear extracts per reaction. Supershift with an anti-p50 antibody was also performed. DNA-binding complexes were separated by electrophoresis on a 5% polyacrylamide-Tris/glycine-EDTA gel, which was dried and exposed to X-ray film.

### Effect of PPS on TNF $\alpha$ -Induced Increase in Albumin Permeability in Podocytes

In all,  $1 \times 10^5$  podocytes<sup>28</sup> were seeded onto collagen-coated Transwell filters (0.4  $\mu$ M pore-size, Corning, New York, NY, USA) placed in the top chamber of each well of a 24-well plate. After confluence was reached, some wells were treated with TNF $\alpha$  (20 ng/ml), PPS (400  $\mu$ g/ml) or PPS plus TNF $\alpha$  in 2% FBS normal glucose medium for 8 h. Then the medium from both the top and bottom chamber was completely removed, cells were washed twice with PBS and the top chamber was refilled with 0.2 ml 2% FBS medium supplemented with 0.5 mg/ml FITC-labeled bovine serum albumin

(BSA) (Sigma Aldrich, St Louis, MI, USA), while the bottom chamber was refilled with 0.6 ml 2% FBS medium supplemented with 0.5 mg/ml unlabeled BSA. At different time points, 100  $\mu$ l aliquots were collected from the bottom chamber and fluorescence was measured by a fluorescence spectrophotometer (485 nm excitation, 535 nm emission). The concentration of FITC-BSA passing through the monolayer was determined by reference to a set of standard dilutions of FITC-BSA. The value of albumin flux through untreated monolayer was arbitrarily defined as 100%.

### Effect of PPS on High Glucose and AGE-Stimulated MCP-1 Production in Podocytes

A podocyte cell line was used for the experiments.<sup>28</sup> In all,  $1 \times 10^4$  cells were seeded in each well of a six-well-plate. Cells were cultured under 30 mM high glucose in the presence or absence of PPS (200  $\mu$ g/ml) for 3 days. Cells in 6 mM normal glucose were used as control. As AGEs formed from the interaction between high glucose and proteins have been shown to have an important role in high glucose-mediated inflammation, we also tested the effect of PPS on AGE-stimulated MCP-1 production in podocytes. Cells were treated with AGEs derived from BSA (AGE-BSA, 200  $\mu$ g/ml) with or without PPS (200  $\mu$ g/ml) for 24 h. Cells treated with BSA served as control. MCP-1 in cell supernatants was determined by ELISA and corrected by cell number. NF- $\kappa$ B transcription activity was also examined by reporter assay as described above in podocytes treated with AGEs with or without PPS.

### Statistical Analysis

Values were expressed as mean  $\pm$  s.d. ANOVA or two-tailed unpaired *t*-test was used to evaluate the differences between the means. Significance was defined as  $P < 0.05$ .

## RESULTS

### General

Body weight was not significantly reduced in old diabetic mice (Table 1). The heart weight/body weight or kidney weight/body weight ratio in old diabetic mice remained

**Table 1** General characteristics of diabetic mice

	Old mice (22 months)		
	Control (n = 10)	Diabetic (n = 11)	PPS (n = 10)
Body weight (g)	25.7 $\pm$ 1.4	24.4 $\pm$ 0.5	24.1 $\pm$ 1.3
Heart weight (mg)	167 $\pm$ 19	169 $\pm$ 23	170 $\pm$ 14
Kidney weight (mg)	218 $\pm$ 11	225 $\pm$ 19	221 $\pm$ 23
Glucose (mg/dl)	106 $\pm$ 34	301 $\pm$ 36	332 $\pm$ 68
Scr (mg/dl)	0.109 $\pm$ 0.03	0.138 $\pm$ 0.02*	0.118 $\pm$ 0.02

PPS, pentosan polysulfate

\* $P < 0.05$  vs old mice control.

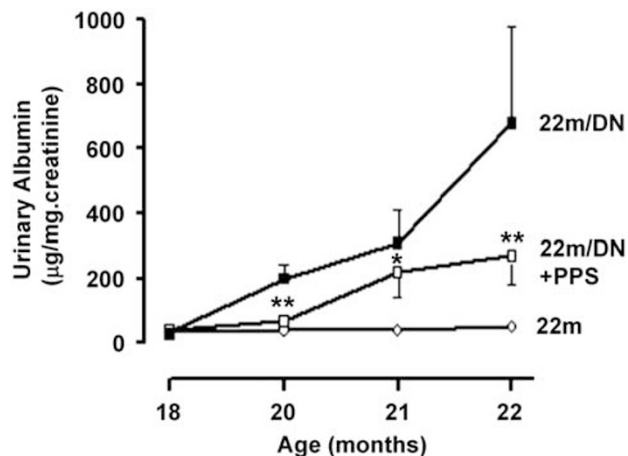
similar to their age-matched controls, and PPS treatment did not change these values. There was no significant difference in glycemic levels between PPS-treated and -untreated old diabetic mice. No ketonuria was spotted in both groups of diabetic mice. However, serum creatinine levels were significantly elevated in untreated 22-month-old diabetic mice as compared with age-matched non-diabetic mice ( $P < 0.05$ ) (Table 1). PPS treatment prevented the increase in serum creatinine.

### Albuminuria

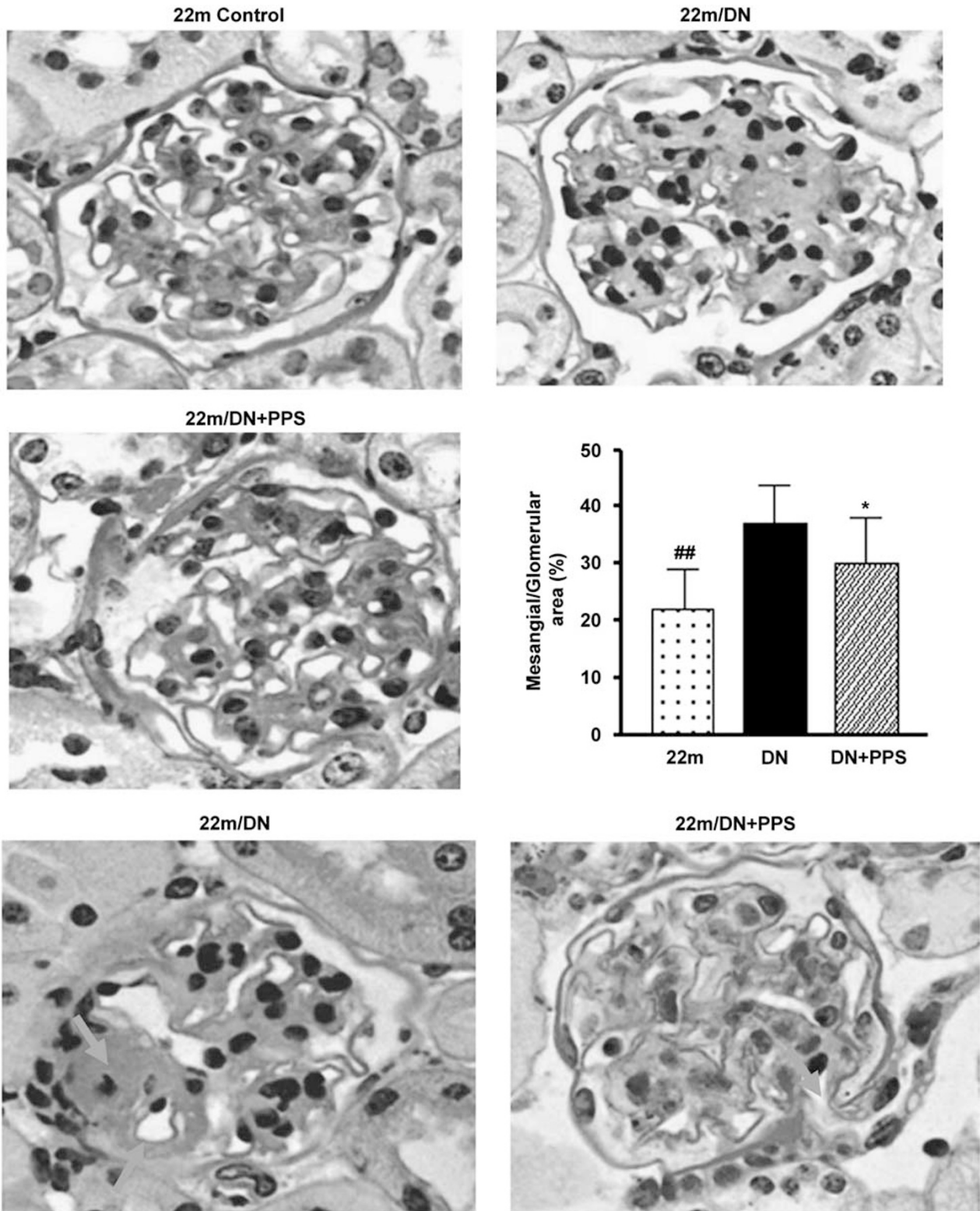
Old diabetic mice developed progressively severe albuminuria. Urinary albumin excretion was 7-fold higher after 2 months of diabetes, and continued to increase after 4 months of diabetes, reaching levels which were about 14-fold higher, than in old control mice (Figure 1). The increase in albuminuria was blunted in PPS-treated old diabetic mice, reaching a plateau after 3 months of diabetes, and remained lower than untreated old diabetic mice.

### Histopathology

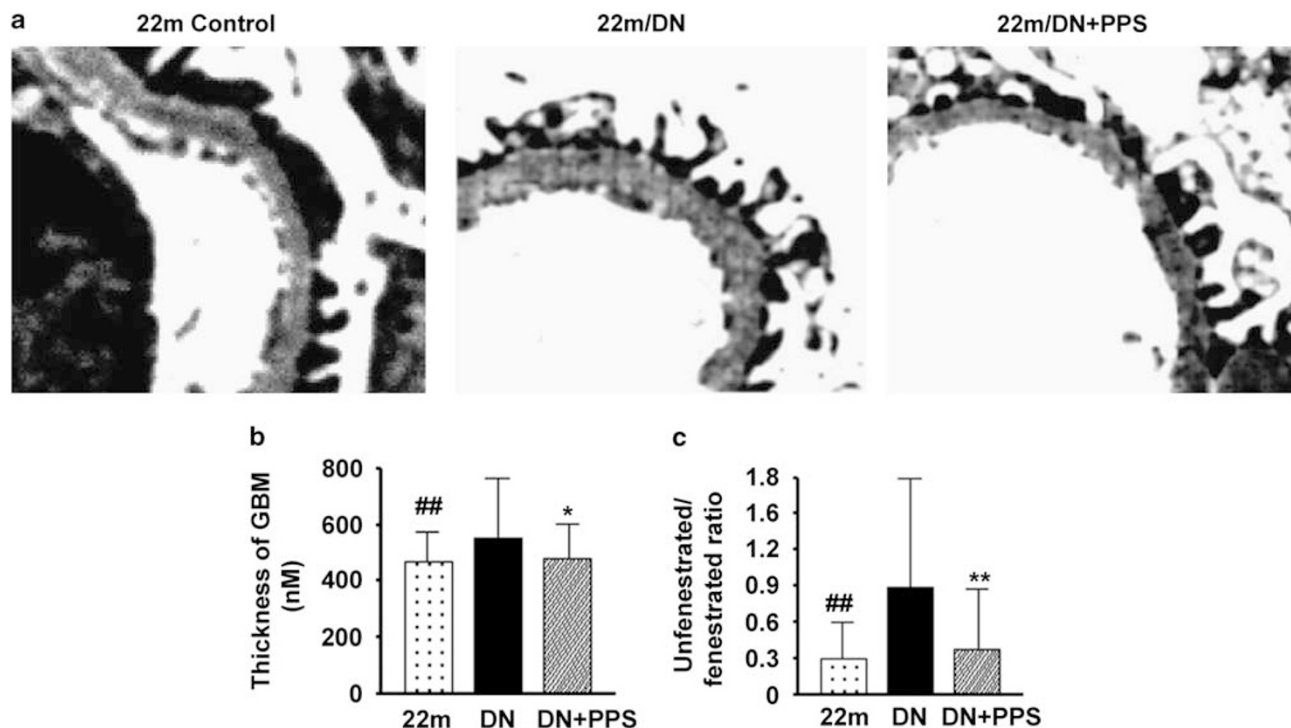
As previously reported, 22-month-old non-diabetic control mice had increased glomerular size, a slight increase in the mesangial areas, and a largely normal tubulointerstitium<sup>20,24</sup> (Figures 2 and 3). The superimposition of diabetes resulted in severe glomerular, tubulointerstitial and vascular lesions<sup>24</sup> (Figures 2 and 3). There was an increase in overall glomerular cell number in old diabetic mice ( $49.5 \pm 4.9$  nuclei/glomerulus vs  $43.7 \pm 8.5$  nuclei/glomerulus in 22-month-old controls,  $P < 0.05$ ). The mesangium was diffusely and markedly increased (Figure 2). Morphometric analysis confirmed that mesangial area was significantly enlarged in old diabetic mice compared with 22-month-old controls ( $P < 0.05$ ) (Figure 2). Typical vascular lesions seen in human DN including the



**Figure 1** Decreased albuminuria in PPS-treated aging diabetic mice. Twenty-two-month-old diabetic mice developed progressive albuminuria (22 month/DN), which was significantly reduced by PPS treatment. \* $P < 0.05$ , \*\* $P < 0.01$  vs untreated diabetic mice at the same time point. Albuminuria was not present in 22-month-old non-diabetic control mice.



**Figure 2** Reduced glomerular lesions in PPS-treated aging diabetic mice. Representative pictures show that compared with 22 months control mouse, mesangial area is enlarged in 22-month-old diabetic mouse (22 month/DN, PAS  $\times 400$ ). Mesangial expansion is reduced in PPS-treated diabetic mouse (22 month/DN + PPS, PAS  $\times 400$ ). Morphometry analysis of mesangial area, expressed as a percentage of total glomerular area, was increased in the kidneys of untreated diabetic mice (DN).  $##P < 0.01$  vs 22-month controls. PPS treatment decreased mesangial area in diabetic mice.  $*P < 0.05$  vs DN. Arteriolar lesions characterized by hyalinosis are present in afferent and efferent arterioles (arrows) of untreated 22-month-old diabetic mouse while the arteriole is virtually normal in PPS-treated mouse (arrow).



**Figure 3** Reduction in the thickness of glomerular basement membranes and increased glomerular endothelial fenestration in PPS-treated aging diabetic mice. (a) The basement membranes are irregularly thickened and there is fusion of foot processes in 22-month-old diabetic mice (EM  $\times$  5000). The thickness of basement membrane is reduced and podocyte foot processes are relatively well preserved in PPS-treated aging diabetic mice. (b) Measurement of the width of the glomerular basement membranes showed increased thickness in untreated 22-month-old diabetic mice (## $P$  < 0.01 vs 22-month controls). PPS-treatment decreased the thickness. \* $P$  < 0.05 vs untreated aging diabetic mice (DN). (c) Quantitative analysis of glomerular endothelial cell fenestration. Data are expressed as the ratio of unfenestrated to fenestrated glomerular endothelia. The unfenestrated areas were significantly increased in glomeruli of untreated aging diabetic mice. ## $P$  < 0.01 vs 22-month controls. The unfenestrated areas were reduced by PPS treatment. \*\* $P$  < 0.01 vs untreated aging diabetic mice (DN). The glomerular basement membranes are relatively normal in 22-month-old control mouse (EM  $\times$  5000).

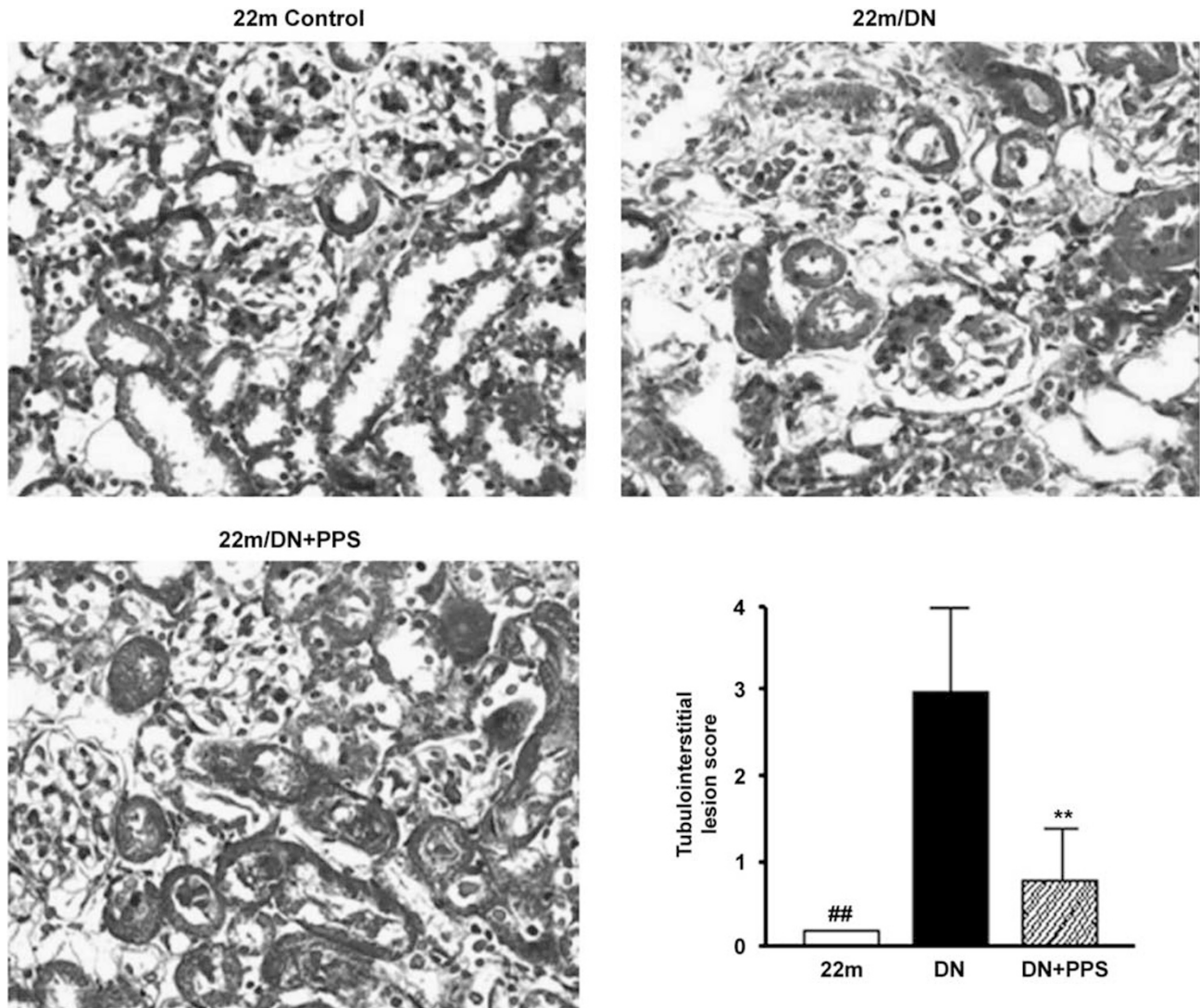
increase in thickness of arteriolar wall and hyaline deposition in afferent and efferent arterioles were present in old diabetic mice (Figure 2). The glomerular basement membranes were generally thickened in old diabetic mice, with some areas of marked increase in width by electron microscopy (Figure 3). Effacement of podocyte foot processes was present in some peripheral loops and the number of glomerular capillary endothelial cells in fenestrae was significantly decreased in old diabetic mice ( $P$  < 0.05) (Figure 3). There was diffuse tubular atrophy, interstitial fibrosis and inflammatory cell infiltrate in old diabetic mice ( $P$  < 0.01) (Figure 4).

The kidney lesions were significantly improved by PPS treatment. The expansion of mesangial areas was reduced ( $P$  < 0.05) (Figure 2). The number of glomerular cells was decreased ( $42.8 \pm 12.3$  vs  $49.5 \pm 4.9$  nuclei/glomerulus in untreated diabetic mice) ( $P$  < 0.01). In addition, the thickness of basement membranes and the loss of glomerular endothelial fenestrations were decreased by PPS-treatment (Figure 3). There were no hyalinosis and obvious vascular wall lesions in PPS-treated old diabetic kidneys (Figure 2). Importantly, PPS treatment nearly completely prevented the development of tubulointerstitial lesions in old diabetic mice

( $P$  < 0.01) (Figure 4). As tubules are actively involved in albumin retrieval and degradation, tubular injury has been suggested to have a role in albuminuria.<sup>29–31</sup> Thus, we examined albumin staining in kidneys from old controls and old diabetic mice treated with or without PPS. Albumin-positive staining was present in some of glomerular parietal epithelial and tubular cells in old control mice (Figure 5). A prominent increase in staining intensity in tubules was found in old diabetic mice. Digital quantitation of the intensity of immunostaining supported the visual impression of increased tubular albumin staining in old diabetic kidneys. PPS treatment largely prevented the increase (Figure 5).

#### PPS Treatment Decreased Macrophage Infiltrates in the Kidneys of Old Diabetic Mice

Kidney sections were stained with an anti-F4/80, a macrophage marker, to characterize the inflammatory lesions. Macrophages were rarely seen in the kidneys of control old mice (Figure 6a). There was an extensive infiltrate of macrophages and it was common to see macrophage cluster in untreated kidneys of old diabetic mice (Figure 6b). Direct measurement of macrophage positively stained area showed



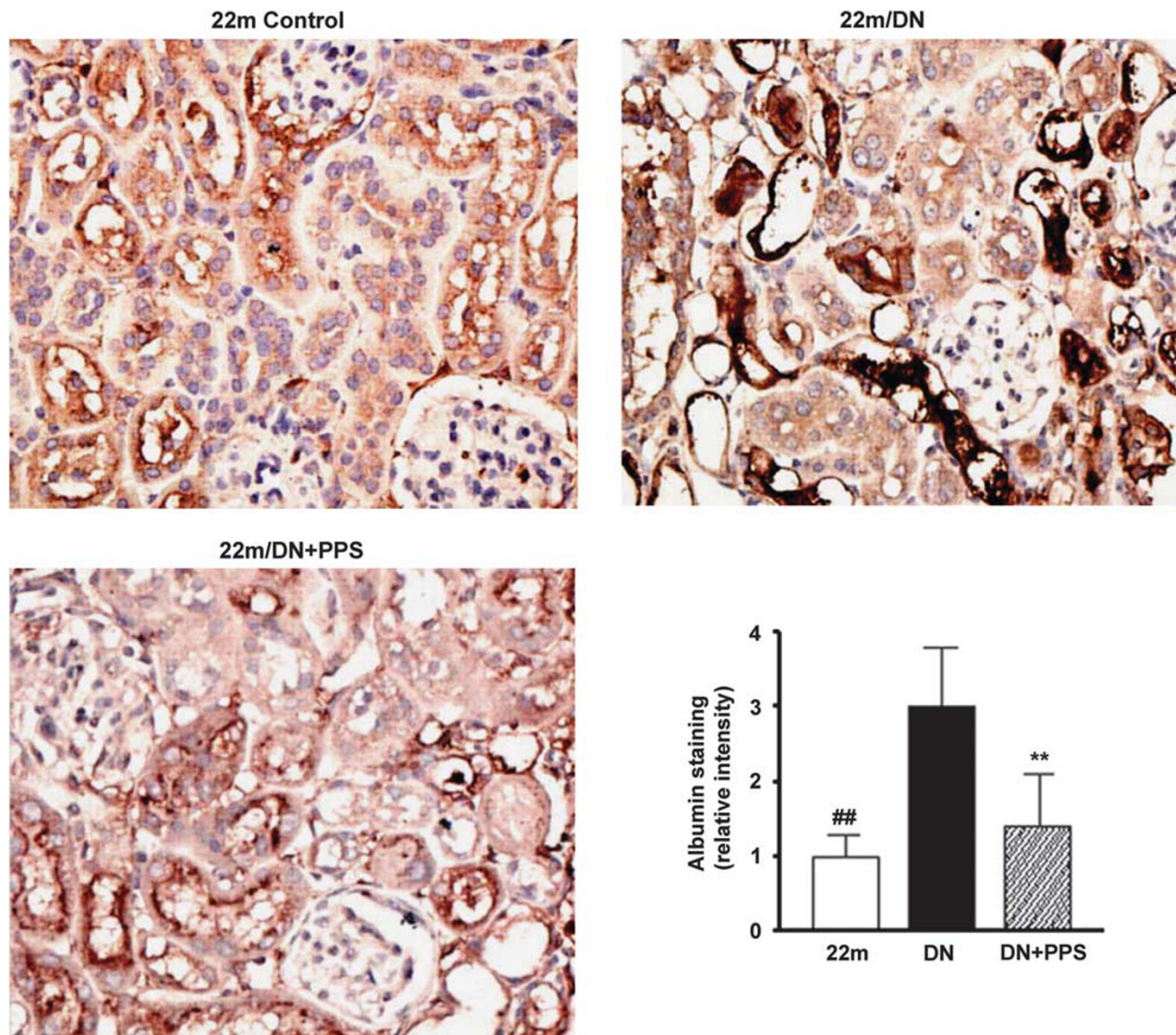
**Figure 4** Prevention of tubulointerstitial lesions by PPS treatment in aging diabetic mice. Although tubulointerstitium is largely normal in 22 months control mice, untreated 22-month-old aging diabetic mice have severe tubulointerstitial lesions including extensive inflammatory cell infiltration and diffuse tubular atrophy and interstitial fibrosis (blue staining of matrix), representative Masson's trichrome stains (22 month/DN, 200 ×). The tubulointerstitial lesions were markedly decreased in PPS-treated mice (22 month/DN + PPS), as evidenced by decreased number and size of connective tissue zones (blue staining) and a sharp reduction in the amount of inflammatory cells in the interstitium. The tubulointerstitial lesions in untreated aging diabetic mice (DN), assessed by a semiquantitative measurement was significantly greater than in PPS-treated aging diabetic mice (DN + PPS) (3 + vs 0.8 +). ##*P* < 0.01, \*\**P* < 0.01 vs untreated aging diabetic mice. The tubules and interstitium in kidneys from 22-month-old non-diabetic control mice were essentially normal.

that about 10% of tubulointerstitium in untreated old diabetic mice was occupied by macrophages (Figure 6). The number of infiltrating macrophages was substantially reduced after PPS treatment (Figure 6c). Although nuclear phosphorylated NF- $\kappa$ B p65 staining, an indication of NF- $\kappa$ B activation, was present in interstitial capillary endothelial cells, parietal epithelial cells, and some cells of distal tubules and collecting ducts in the kidneys of 22-month-old non-diabetic mice<sup>24</sup> (Figure 6d), the number of NF- $\kappa$ B-positive cells was markedly increased in the glomeruli and tubulointerstitium of untreated old diabetic mice (41 ± 21 cells per field vs 16 ± 9 cells per field in old controls, *P* < 0.01,

Figure 6). This number was sharply decreased by PPS treatment (Figure 6).

There was a significant increase in mRNA levels of MCP-1, RANTES and CXCL-1 in the kidneys of untreated old diabetic mice (Figures 7a–c), but PPS treatment decreased the upregulation of these chemokines (*P* < 0.01). The expression of IL-6 and TNF $\alpha$ , two cytokines that are closely associated with chronic inflammation in aging and have an active role in inflammation were examined to explore their role in the kidneys of old diabetic mice. IL-6 mRNA levels were similar between the kidneys of old diabetic and control mice (Figure 7d), and PPS treatment did not affect the levels.





**Figure 5** Immunostaining for albumin. Positive staining for albumin is seen in parietal epithelial cells of glomerulus and some tubules of 22 months control mice. There is a visible increase in albumin staining intensity in tubules of untreated aging diabetic mice. Albumin staining in tubules of PPS-treated aging diabetic mice is similar to that in old controls. The intensity of albumin staining was measured by a morphometry software and defined by an arbitrarily unit. ## $P < 0.01$ , \*\* $P < 0.01$  vs untreated aging diabetic mice.

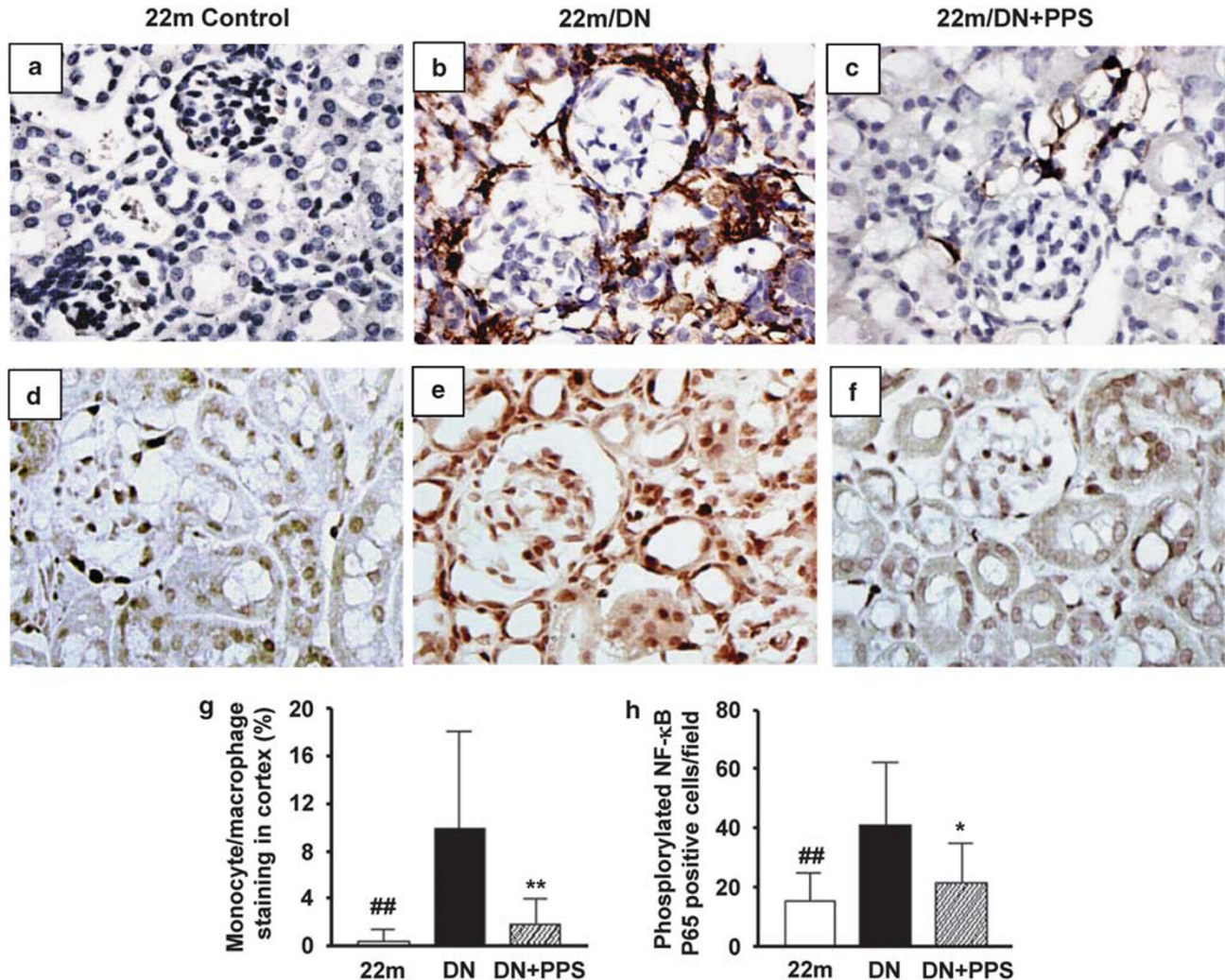
Although TNF $\alpha$  mRNA was not expressed in 22-month-old control kidneys, it was present in untreated old diabetic kidneys<sup>24</sup> (Figure 6e). The expression of TNF $\alpha$  mRNA was not demonstrable in the kidneys of old diabetic mice treated with PPS (Figure 6e). Moreover, the levels of MCP-1, RANTES and CXCL-1 mRNA were decreased in PPS-treated old diabetic mice, coincident with the reduction of infiltrating macrophages, lower levels of NF- $\kappa$ B activation and the absence of TNF $\alpha$  expression (Figures 6 and 7).

#### Inhibition of TNF $\alpha$ Action by PPS via Suppression of NF- $\kappa$ B

To determine whether PPS directly affected the pro-inflammatory action of TNF $\alpha$ , proximal tubular cells were

pretreated with PPS before exposure to TNF $\alpha$ . PPS largely blocked TNF $\alpha$ -induced upregulation of MCP-1, RANTES, MIP-2, ICAM-1 and iNOS in proximal tubular cells (Figures 7f–m). As both the NF- $\kappa$ B and MAPKs pathways are critically involved in proinflammatory action of TNF $\alpha$ , we examined the effect of PPS on NF- $\kappa$ B and MAPK activation after TNF $\alpha$ -stimulation. Phosphorylated I $\kappa$ B levels were increased at 30 min in proximal tubular cells after TNF $\alpha$  stimulation and remained high after 2 h (Figure 8a). When cells were treated with PPS before TNF $\alpha$ -stimulation, the levels of I $\kappa$ B phosphorylation were decreased 40–60%. However, the levels of total I $\kappa$ B seem to be stable in proximal tubular cells even after TNF $\alpha$  stimulation. Thus, we further assessed NF- $\kappa$ B transcription activity. The NF- $\kappa$ B luciferase reporter assay





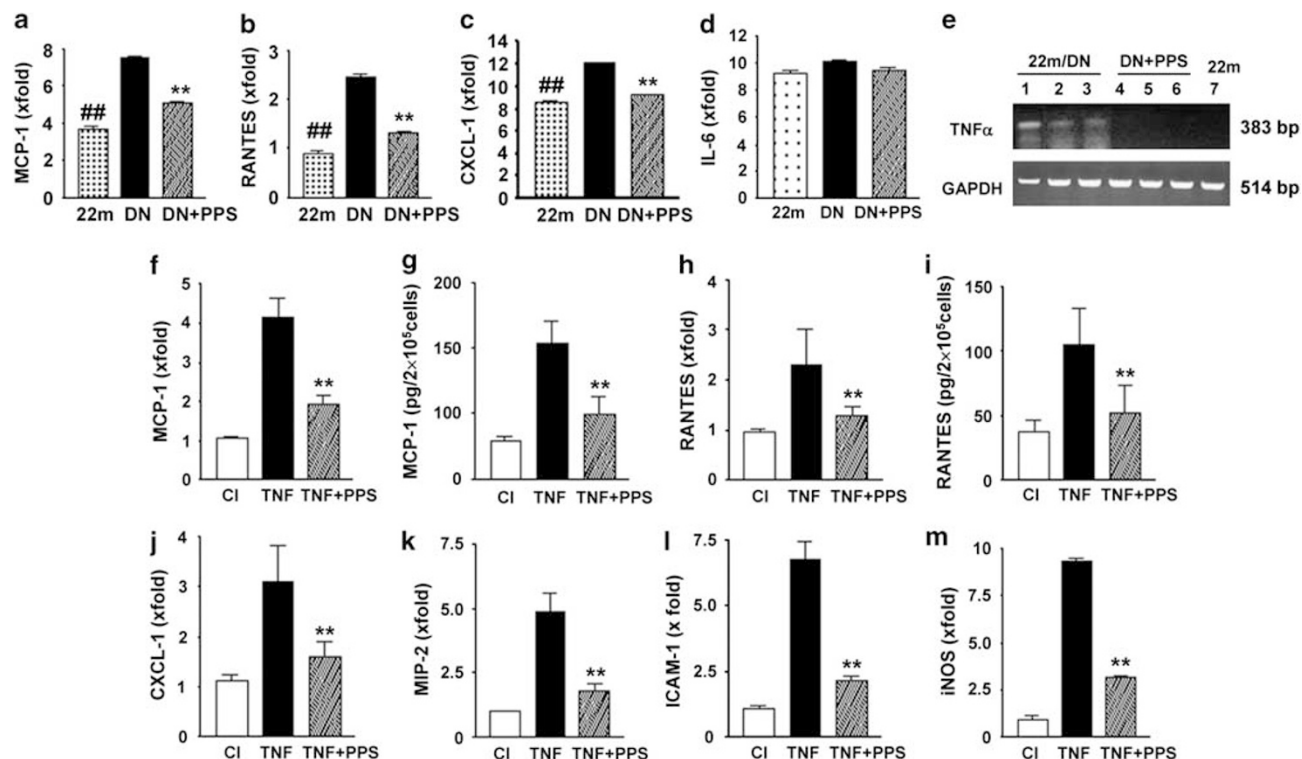
**Figure 6** Immunostaining for monocyte/macrophage and NF- $\kappa$ B. The presence of macrophages was assessed by F4/80 staining. Positive staining is rarely found in 22-month-old control mice (a). Diffuse and multi-focal areas of macrophage infiltration are present in the tubulointerstitium of 22-month aging diabetic mice (22 month/DN, b). The number of macrophages was substantially reduced in PPS-treated mice (22 month/DN + PPS, c). Some nuclei in glomerular parietal epithelials and in glomerulus are stained positively for phosphorylated NF- $\kappa$ B (p65) (Ser276), a marker of NF- $\kappa$ B activation ( $\times 500$ , d). A prominent increase in NF- $\kappa$ B-positive cells is seen in the glomerulus, tubules and interstitium of aging diabetic mice (e). The number of nuclei staining positively for NF- $\kappa$ B positive is noticeably decreased in PPS-treated aging diabetic mice (f). Quantitative measurement of area occupied by monocytes/macrophages shows that an average of 10% renal cortex in aging diabetic mice are these cells (g). ## $P < 0.01$ , \*\* $P < 0.01$  vs untreated aging diabetic mice (DN). (h) The number of NF- $\kappa$ B-positive nuclei was counted in sections from 22-month control, aging diabetic and PPS-treated aging diabetic mice. ## $P < 0.01$ , \* $P < 0.05$  vs untreated aging diabetic mice (DN).

showed that PPS treatment dose-dependently decreased TNF $\alpha$ -stimulated NF- $\kappa$ B transcriptional activity in proximal tubular cells (Figure 8b). Furthermore, PPS treatment nearly completely blocked the TNF $\alpha$ -induced increase in NF- $\kappa$ B DNA-binding activity (Figure 8c). PPS treatment also suppressed TNF $\alpha$ -stimulated ERK1/2 phosphorylation in proximal tubular cells (data not shown).

#### High Glucose- and AGE-Stimulated MCP-1 Production in Podocytes is Decreased by PPS Treatment

Since hyperglycemia could either directly increase inflammation, or do so via the formation of AGE, we assessed the

levels of MCP-1 in podocytes at baseline and after exposure to high glucose or AGE in the presence or absence of PPS. Although exposure to 30mM high glucose for 3 days resulted in increased MCP-1 production in podocytes, the increase was abrogated by PPS (Figure 9a). Additionally, podocytes cultured in 6mM glucose also had decreased MCP-1 production when PPS was added to the medium. As previously reported, AGE stimulation increased MCP-1 production and activated NF- $\kappa$ B transcription activity in podocytes<sup>32</sup> (Figures 9b and c). Both baseline and AGE-stimulated NF- $\kappa$ B transcription activity and MCP-1 production was suppressed by PPS treatment (Figure 9).



**Figure 7** PPS treatment decreased expression of proinflammatory genes. (a–c) MCP-1, RANTES and CXCL-1 mRNA levels were significantly increased in aging diabetic mice, compared with aging control mice. The increase was largely blocked by PPS treatment. The MCP-1, RANTES and CXCL-1 mRNA levels in 22-month-old control (22 month) and 22-month-old diabetic mice treated without (DN) and with PPS (DN+PPS) were determined by real-time PCR and were corrected by  $\beta$ -actin mRNA levels in the same sample.  $**P < 0.01$  vs untreated aging diabetic mice (DN). (d) IL-6 mRNA expression: IL-6 mRNA levels in kidneys were determined by real-time PCR. There was no difference in IL-6 mRNA levels among 22-month-old non-diabetic and aging diabetic mice treated with and without PPS. (e) Representative gels of  $\text{TNF}\alpha$  and GAPDH mRNA levels of kidneys from 22-month-old, 22-month-old diabetic mice (22 month/DN) and 22-month-old diabetic mice treated with PPS (22 month/DN + PPS).  $\text{TNF}\alpha$  mRNA expression was only found in the kidneys of 22-month-old diabetic (lanes 1–3). The expression was absent in 22-month-old (lane 7) and PPS-treated 22-month-old diabetic mice (lanes 4–6) even when PCR cycles were increased to 40. cDNA obtained from mouse macrophages (*Mφ*) was used as a positive control for PCR (data not shown). The levels of GAPDH mRNA were relative similar among the samples. (f–m) PPS decreased  $\text{TNF}\alpha$ -stimulated inflammation in proximal tubular cells. Proximal tubular cells were treated with PPS (200  $\mu\text{g}/\text{ml}$ ) or with saline for 1 h before stimulating with  $\text{TNF}\alpha$  (10 ng/ml). RNA was collected 4 h later. The mRNA levels in cells without  $\text{TNF}\alpha$  stimulation (control) were arbitrarily defined as 1.  $\text{TNF}\alpha$  treatment caused a 2–9-fold increase in the expression of the following mRNAs: (f) MCP-1, (h) RANTES, (j) CXCL-1, (k) MIP-2, (l) ICAM-1 and (m) iNOS. Pretreatment with PPS largely prevented the increased expression of these mRNAs after  $\text{TNF}\alpha$  treatment. Furthermore, PPS decreased  $\text{TNF}\alpha$ -stimulated (g) MCP-1 and (i) RANTES protein production by proximal tubular cells.  $**P < 0.01$  vs  $\text{TNF}\alpha$  alone.

### **$\text{TNF}\alpha$ -Induced Increased Albumin Permeability in Podocytes *In Vitro* is Decreased by PPS Treatment**

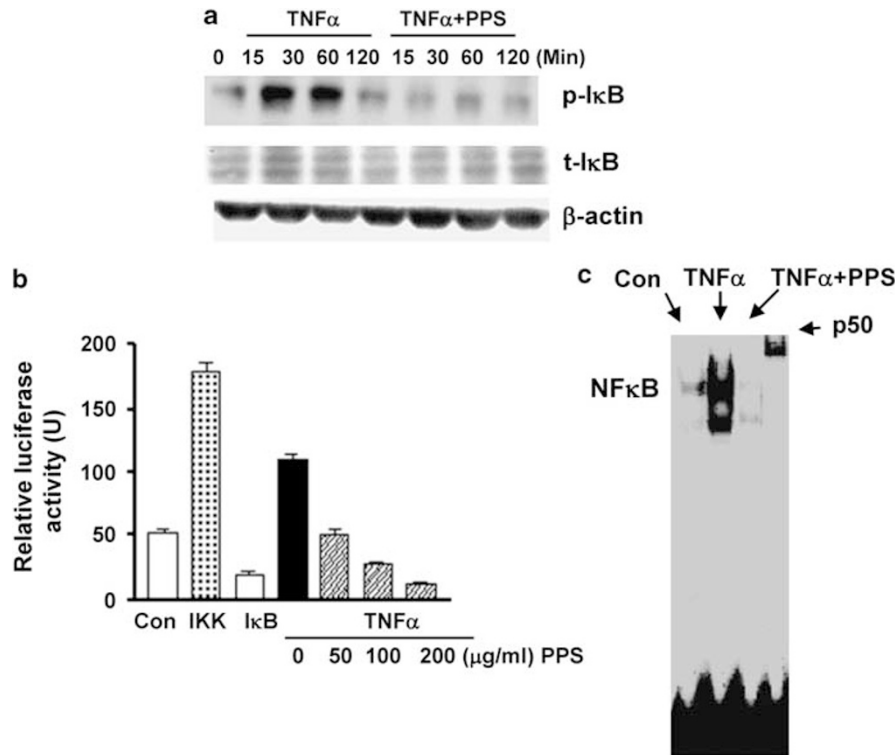
The amount of FITC-labeled albumin that passed through a monolayer of podocytes was increased by 34% at 2 h after  $\text{TNF}\alpha$  treatment. This increase was reduced by nearly 60% in the presence of PPS (Figure 10).

### **DISCUSSION**

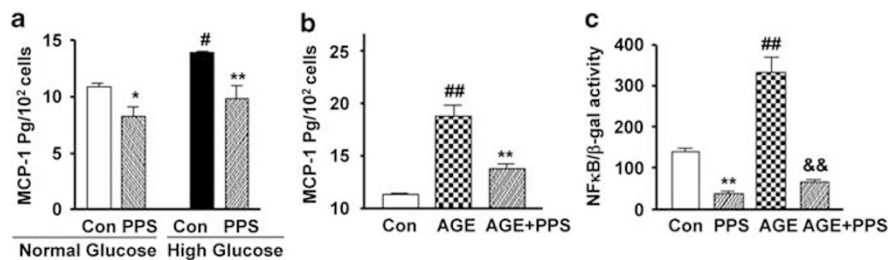
Although the pathogenesis of DN is complex, it is increasingly regarded as an inflammatory disease.<sup>7,33–35</sup> This conclusion is largely based on the presence of lymphocyte and macrophage infiltration and overexpression of proinflammatory cytokines and chemokines in kidney at every stage of disease in human. Moreover, inflammation very likely has a role in the progression of DN, because mice lacking MCP-1 or ICAM-1 are resistant to DN.<sup>36–39</sup> Although

several anti-inflammatory drugs have been proposed for the treatment of DN,<sup>12,40–42</sup> the lack of a DN model with typical inflammatory lesions has hindered their study. We recently established a model of DN in aging mice that mimics DN in humans,<sup>24</sup> making it possible to address the possibility that the anti-inflammatory properties of PPS might be useful for the treatment of DN. Diabetic aging C57B6 mice develop severe nephropathy with renal insufficiency, progressive albuminuria, and extensive glomerular, vascular and tubulointerstitial lesions. We found that PPS treatment substantially decreased inflammation and the progression of DN in aging mice.

The inflammatory lesions in aging DN were characterized by extensive macrophage infiltration and upregulation of  $\text{TNF}\alpha$ , a proinflammatory cytokine, as well as several chemokines (MCP-1, RANTES and CXCL-1).<sup>24</sup> As  $\text{TNF}\alpha$  is actively



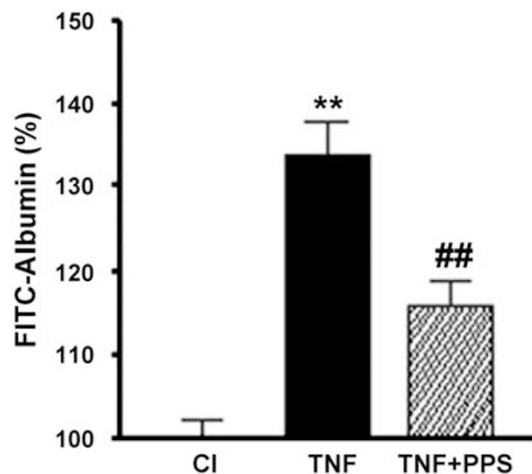
**Figure 8** (a) TNF $\alpha$ -stimulated NF- $\kappa$ B activation is suppressed by PPS. Proximal tubular cells were pretreated with PPS or saline for 30 min before the addition of TNF $\alpha$ . Cellular protein was collected the indicated time points. Phosphorylated I $\kappa$ B, total I $\kappa$ B and  $\beta$ -actin levels were determined by western blot. The experiments were repeated three times. (b) NF- $\kappa$ B transcription activity was determined by transfecting proximal tubular cells with a luciferase reporter in the presence or absence of an IKK or I $\kappa$ B cDNA. Transfection efficiency was determined by co-transfecting cells with a  $\beta$ -galactosidase plasmid. Data are expressed as relative units after correcting by the galactosidase activity. Transfected cells were also treated with TNF $\alpha$  in the presence or absence of PPS after transfection. (c) Nuclear protein was isolated from proximal tubular cells 60 min after treating cells with TNF $\alpha$  in the presence or absence of PPS. The binding of NF- $\kappa$ B protein to DNA was determined by a gel shift assay. Positive controls included macrophages treated with LPS and the presence of anti-p50 antibody in the reaction. NF- $\kappa$ B DNA-binding activity is weakly present in untreated proximal tubular cells (control) and the activity is increased by TNF $\alpha$ . TNF $\alpha$ -stimulated NF- $\kappa$ B DNA-binding activity is nearly completely blocked by PPS treatment. The presence of p50 antibody visibly reduces the mobility.



**Figure 9** PPS suppressed high glucose and AGE-stimulated inflammation in podocytes. (a) Podocytes were grown in high glucose (25 mM) and normal glucose (6 mM) in the presence or absence of PPS (200  $\mu$ g/ml) for 3 days. MCP-1 production by the cells was determined. \* $P$ <0.05 vs control (con); # $P$ <0.05 vs cells cultured in 6 mM glucose; \*\* $P$ <0.01 vs cells cultured in 25 mM glucose. (b) Podocytes were treated with AGE-BSA (200  $\mu$ g/ml) in the presence or absence of PPS (200  $\mu$ g/ml) for 24 h. Control (Con) were cells treated with BSA (200  $\mu$ g/ml). ## $P$ <0.01 vs Control; \*\* $P$ <0.01 vs cells treated only with AGE. (c) PPS decreased AGE-stimulated NF- $\kappa$ B transcription activity. Activity was determined by transfecting podocytes with a luciferase reporter. Cells were treated with AGE (200  $\mu$ g/ml) in the presence or absence of PPS (200  $\mu$ g/ml). Transfection efficiency was determined by co-transfecting cells with a  $\beta$ -galactosidase plasmid. Data are expressed as relative units after correcting by galactosidase activity. \*\* $P$ <0.01, ## $P$ <0.01 vs Control (Con); && $P$ <0.01 vs cells treated only with AGE.

involved in chronic inflammatory diseases and induces proinflammatory responses in glomerular and tubular cells both *in vitro* and *in vivo*,<sup>24,43–45</sup> the upregulation of TNF $\alpha$  in

aging diabetic kidney may contribute to the inflammatory lesions. In this study, we found that PPS suppresses TNF $\alpha$  expression and TNF $\alpha$ -mediated inflammation. Renal TNF $\alpha$



**Figure 10** PPS decreases TNF $\alpha$ -stimulated albumin permeability in podocyte monolayers. A monolayer of podocytes was created on collagen-coated Transwell filters in a 24-well plate. Wells were treated with TNF $\alpha$  (20 ng/ml), PPS (400  $\mu$ g/ml) or PPS plus TNF $\alpha$ . FITC-labeled BSA was added to the cells and the amount of FITC-labeled BSA that passed through the cell layer was quantitated. Negative controls included unlabeled BSA or filters without cell layers. The experiments were repeated three times. Results shown are fluorescence intensity 2 h after the addition of FITC-BSA. The value of albumin flux through monolayer without TNF $\alpha$  or PPS treatment was arbitrarily defined as 100%. \*\* $P < 0.01$  vs control; ## $P < 0.01$  vs cells treated only with TNF $\alpha$ .

mRNA expression in aging diabetic mice was decreased by PPS treatment. Moreover, TNF $\alpha$ -induced upregulation of proinflammatory genes including MCP-1, RANTES, CXCL-1, MIP-2 and iNOS in proximal tubular cells and podocytes was largely blocked by PPS. As PPS decreased TNF $\alpha$ -stimulated I $\kappa$ B phosphorylation and NF- $\kappa$ B activation, this may be one mechanism of its suppression of TNF $\alpha$  action. Finally, PPS also decreased high glucose and AGE-mediated MCP-1 production and NF- $\kappa$ B activation. Both hyperglycemia and elevated AGE are critically involved in inflammation in diabetes.

We observed extensive macrophage infiltration in kidneys of aging diabetic C57B6 mice. This was nearly completely prevented by PPS treatment. Macrophage depletion experiments have shown that macrophages are important in the development and progression of diabetic complications including nephropathy.<sup>33</sup> Infiltrating macrophages may contribute to tissue damage and lead to fibrosis through the production of oxidants and release of proteases and cytokines.<sup>46,47</sup> Although it is unclear how PPS treatment decreases macrophage infiltration, the reduction of macrophages may contribute to the effect of PPS on glomerular and tubulointerstitial lesions in aging diabetic mice.

Albuminuria, a hallmark of DN, is an independent risk factor for progressive renal structural damage.<sup>29,48</sup> Although increased TNF $\alpha$  has been proposed to be correlated with the degree of albuminuria in DN,<sup>49</sup> there is no direct evidence of TNF $\alpha$  for this at present. The current findings that

TNF $\alpha$ -treatment increased the permeability of podocytes monolayers on Transwell filters to albumin, provides some support for this postulate. The fact that PPS decreased the TNF $\alpha$ -mediated increase in albumin permeability in the podocyte monolayers, suggests that this may be one action accounting for the reduction of albuminuria by PPS. Tubular injury may also have an important role in albuminuria. In this study, we have also observed intensive albumin staining in tubules of aging diabetic mice. As PPS-treated aging diabetic mice have visibly decreased tubular albumin staining, a protection against tubular injury may also contribute to decreased albuminuria in PPS-treated mice.

We have found that C57B6 mice develop a slowly progress kidney aging that begins at 18–20 months of age. The obvious albuminuria and renal fibrosis are present at 28–30 months of aging. Additional injury such as diabetes accelerates the process of kidney aging in C57B6 mice. In this study, PPS was shown to decrease diabetic injury in 22-month-old mice. It would be interesting for future experiments to examine if PPS decrease the process of normal kidney aging.

In conclusion, this study shows that PPS treatment prevents the progression of nephropathy in streptozotocin-induced diabetes in aging C57B6 mice by decreasing albuminuria, renal macrophage infiltration and TNF- $\alpha$  expression. These effects were associated with improvement of histopathologic changes and renal function. Although the mechanisms of action may be complex, PPS suppresses inflammation mediated by TNF $\alpha$ , high glucose and AGEs. Since PPS has been widely used for the treatment of interstitial cystitis for nearly 20 years, and has few major side effects, it may be an additional agent that is worthy of further investigation for the treatment of DN. PPS has been shown to be with an excellent long-term safety profile. A 2-year study by National Toxicology Program shows no obvious toxicity in mice fed with PPS below 504 mg/kg/day ([http://ntp.niehs.nih.gov/ntp/htdocs/LT\\_rpts/tr512.pdf](http://ntp.niehs.nih.gov/ntp/htdocs/LT_rpts/tr512.pdf)). Thus, although from pharmacokinetic point of view that the bioavailability of PPS as an oral medicine is low, the wide window of its dosage range may be sufficient to offset this limit.<sup>50</sup>

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#### DISCLOSURE/CONFLICT OF INTEREST

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