Toll-like receptor 2 agonist exacerbates renal injury in diabetic mice

FANGLIN LI1,2, NINGYU ZHANG2, ZHIMING LI2, LIHUA DENG2, JIANJIE ZHANG2 and YUNFENG ZHOU1

1AstraZeneca-Shenzhen University Joint Institute of Nephrology, Shenzhen University Health Science Center, Shenzhen, Guangdong 518060; 2Division of Internal Medicine, Shenzhen Prevention and Treatment Center for Occupational Diseases, Shenzhen, Guangdong 518001, P.R. China

Abstract. Inflammation is implicated in the pathogenesis of diabetic nephropathy (DN). Toll-like receptor 2 (TLR2) is a ligand-activated membrane-bound receptor, which induces an inflammatory response, thus serving a crucial role in the pathogenesis of DN. The present study aimed to determine whether a TLR2 agonist, PamCysSK4, modulates the development of DN. A mouse model of DN was induced using streptozotocin (STZ) and, following the confirmation of hyperglycemia, mice were treated with or without PamCysSK4. Pathological and functional markers, including the activation of nuclear factor (NF)-κB, expression of TLR2, inflammatory infiltration, myeloid differentiation primary response gene 88 and monocye chemoattractant protein-1 were assessed. STZ-treated mice exhibited elevated blood glucose levels and increased serum creatinine levels, which increased further following PamCysSK4 treatment. In addition, PamCysSK4 treatment was observed to increase podocyte foot process formation. Furthermore, STZ-induced renal glomerular sclerosis was significantly exacerbated in PamCysSK4-treated mice. PamCysSK4-treated mice also exhibited increased levels of collagen IV following renal immunostaining, associated with increased macrophage infiltration. Renal expression of TLR2 was markedly elevated in STZ-induced mice; this was further increased in PamCysSK4-treated mice, accompanied by upregulation of proinflammatory genes and activation of NF-κB. This indicates that enhanced renal expression of TLR2 is associated with inflammatory infiltration in DN and demonstrates that renal injury was exacerbated by the TLR2 agonist in diabetic mice.

Introduction

Diabetic nephropathy (DN) is a major complication associated with diabetes and is one of the leading causes of end-stage renal disease (1,2). It has been suggested that metabolic and hemodynamic factors, including high glucose, transforming growth factor-β1, advanced glycation end products and angiotensin II, are associated with the development of DN (3,4). In early diabetic nephropathy, increased expression of collagen IV in the kidney accompanies increased mesangial cell proliferation (5). In addition to these factors, immune-mediated inflammatory processes are involved in the development and progression of DN (6,7).

A subfamily of the pattern recognition receptors, toll-like receptors (TLRs), serve key roles in regulating immune function and inflammation (8). TLRs are predominantly expressed by a subset of immune cells, including macrophages, T and B cells, and natural killer (NK) cells, but are also expressed by a plethora of non-immune cells, including endothelial cells, renal tubular cells, podocytes and mesangial cells (9,10). Their activation by various ligands triggers a signaling cascade leading to cytokine production and the initiation of an adaptive immune response (8). TLR2 and/or TLR4 are actively involved in the development of kidney diseases, such as ischemia-reperfusion injury, cisplatin-induced nephrotoxicity and crescentic glomerulonephritis (11-13). TLR2 and/or TLR4 are also required for the development of DN (14-18). In streptozotocin (STZ)-induced diabetic animal models, TLR2 and TLR4 expression is enhanced, accompanied by the activation of their downstream signaling pathways (14-16). Clinical studies have indicated that TLR2 and TLR4 expression and activity are significantly upregulated in diabetic patients (17,18), indicating that TLR2 and/or TLR4 may represent targets for the prevention of DN. Previous reports showed that TLR2 primarily signals through the MyD88-dependent pathway to induce inflammation (14). And monocyte chemoattractant protein-1 (MCP-1), a proinflammatory gene, has an important role in diabetic nephropathy (19). PamCysSK4, a synthetic bacterial lipopeptide, is a TLR2 agonist (20). A previous study has demonstrated that PamCysSK4 may accelerate the process of nephrotoxic nephritis through a TLR2-dependent mechanism (13). Although TLR2 has been implicated in the pathogenesis and development of diabetes, it...
is unclear whether the TLR2 agonist Pam₃CysSK₄ influences the development of DN through TLR2/MyD88 and MCP-1 pathway. Thus, the present study aimed to investigate whether TLR2 exacerbates DN.

Materials and methods

Animal model. Mice were maintained under pathogen-free conditions at 22-26°C (humidity, 40-60%) with a 12-h light/dark cycle and free access to water and food. Male C57BL/6 mice (8-10 weeks old; weighing 20-25 g; n=18) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd., (Beijing, China). To assess the effects of TLR2 activation on STZ (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany)-induced renal damage, mice were randomly divided into three groups (n=6/group): (i) Control mice (Con); (ii) mice receiving STZ (STZ); (iii) STZ-induced mice treated with TLR2 agonist Pam₃CysSK₄ (TLR2A+STZ). Mice in the STZ and TLR2A+STZ groups were administered STZ in citrate buffer by intraperitoneal injection (50 mg/kg per day for 5 days). Control mice received citrate buffer alone. After 2 weeks, fasting blood glucose was monitored using an Auto-Check meter (Roche Diagnostics, Basel, Switzerland) (21). Following confirmation of hyperglycemia (blood glucose levels >15 mmol/l), TLR2A+STZ mice received intraperitoneal injection of 100 µg Pam₃CysSK₄ (EMC Microcollections GmbH, Tübingen, Germany) in 10 µl dimethyl sulfoxide (DMSO) four times (once a week); whereas Con and STZ mice received 10 µl DMSO alone weekly for four weeks (13). To measure 24-h proteinuria, mice were placed in individual mouse metabolic cages (Tecniplast S.P.A., Buguggiate, Italy) with ad libitum access to water and food. After 4 weeks, mice were sacrificed by intraperitoneal injection of sodium pentobarbital (30 mg/kg; Abbott Laboratories, Chicago, IL, USA) and their kidneys were removed prior to examination by light and electron microscopy, immunohistochemistry, western blot analysis to measure TLR2 or nuclear factor (NF) -κB protein, by real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The present study was approved by the Animal Experimentation Committee of Shenzhen University Health Science Center (Guangdong, China).

Biochemical assessment of blood and urine samples. At the end of the experiment, blood samples were collected from the left ventricle and centrifuged at 5,000 x g for 10 min at 4°C. Plasma concentrations of total cholesterol, albumin, creatinine and urea nitrogen were measured using an autoanalyzer (Hitachi 917; Hitachi, Ltd., Tokyo, Japan). Urinary albumin excretion was assessed using a Mouse Albumin ELISA kit (cat. no. V900933; Sigma-Aldrich; Merck Millipore). The present study was approved by the Animal Experimentation Committee of Shenzhen University Health Science Center (Guangdong, China).

Assessing podocyte foot process production by electron microscopy. Prior to electron microscopic examination, renal cortex samples were cut into 1-mm³ slices on ice, immediately fixed in 2.5% buffered glutaraldehyde for 12-16 h at 4°C and embedded in epoxy resin. Ultrathin sections (0.1-µm thick) were examined by electron microscopy. To study slit diaphragm morphology, images (20 fields per sample) were captured at random fields of view at a final magnification of x10,000. From each mouse, three glomeruli were evaluated on five 10 k images/gomerulus. Electron microscopy images were analyzed using ImageJ (v2.1.4.7; imagej.nih.gov/ij) and the method of measuring podocyte foot process production was adapted from a previous report (22). From each image, the mean of the foot process width (FPW) was calculated as follows: FPW=2x/4x(∑glomerular basement membrane length/∑foot process).

Renal morphology. Kidney samples were fixed in 4% paraformaldehyde overnight at 4°C, then dehydrated in an ascending ethanol series, embedded in paraffin and cut into sections 4-µm thick. Sections were stained with hematoxylin/eosin and examined with a light microscope to evaluate glomerular mesangial expansion.

Immunohistochemistry studies. Immunohistochemistry was performed to detect collagen IV and F4/80. Briefly, the kidney tissues were fixed in 4% paraformaldehyde overnight at 4°C and blocked for 20 min at 37°C with 1% bovine serum albumin (cat. no. sc-9301; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and F4/80, which is a marker of macrophage infiltration, (1:500; cat. no. MCA497; Bio-Rad Laboratories, Inc., Hercules, CA, USA) overnight at 4°C. Sections were examined using a light microscope (Olympus BX50; Olympus Corporation, Tokyo, Japan) by experimental condition-blinded researchers. To quantify the proportional area of staining, 20 randomly-selected views (magnification, x200) were examined in the renal cortex of each slide.

Western blot analysis. Renal tissue was removed for extraction of total or nuclear protein in order to perform a western blot analysis to measure TLR2 or nuclear factor (NF)-κB expression. Actin or fibrillarin were used as controls. Nuclear proteins were isolated using an NE-PER Nuclear and Cytoplasmic Extraction Kit (cat. no. 78833; Pierce Biotechnology; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. Protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce Biotechnology; Thermo Fisher Scientific, Inc.). Equal amounts of proteins (40 µg) from three groups (n=6) were extracted and separated by 10% SDS-PAGE, and the proteins were transferred to a nitrocellulose membrane. The membrane was washed and blocked in 1X PBS with 0.02% Tween-20 (PBST) supplemented with 5% milk for 1 h at room temperature with gentle shaking, then incubated overnight at 4°C with the primary antibody: Anti-NF-κB p65 antibody (1:500; cat. no. sc-8008) or anti-TLR2 antibody (1:500; cat. no. sc-12504; both Santa Cruz Biotechnology, Inc.). The membrane was washed three times for 30 min in PBST and incubated with horseradish peroxidase-conjugated anti-mouse (1:5,000; cat. no. sc-358917) or anti-goat (1:5,000; cat. no. sc-2768; both Santa Cruz Biotechnology, Inc.) secondary antibody for 1 h at room temperature. Following three washes, the membrane was transferred to the Enhanced Chemiluminescence Reagent (Applygen Technologies, Inc., Beijing, China) and exposed to XBT-1 X-ray film (Kodak, Rochester, NY, USA).

RT-qPCR. Total RNA (100 µg) was extracted from the kidneys of three groups (n=6) with TRIzol reagent
Table I. Metabolic and physiologic parameters in in STZ-induced and Pam,CysSK₄-treated mice.

| Variable                  | Con          | STZ          | TLR2A+STZ     |
|---------------------------|--------------|--------------|--------------|
| Body weight, g            | 27.9±1.4     | 23.2±1.6     | 19.1±1.3     |
| Kidney/body weight, g/kg  | 5.92±0.81    | 9.62±1.46    | 10.86±1.24   |
| Blood glucose, mmol/l     | 5.8±0.7      | 26.7±1.2     | 28.9±1.2     |
| Serum creatinine, µmol/l  | 34.6±2.7     | 36.5±3.4     | 41.5±2.9     |
| Blood urea nitrogen, mmol/l| 7.8±1.2     | 9.6±0.7      | 13.6±1.5     |
| Serum albumin, g/l        | 34.5±3.2     | 31.5±2.1     | 27.0±3.9     |
| Serum cholesterol, mmol/l | 2.05±0.39    | 2.94±0.45    | 2.99±0.45    |

Data are presented as mean ± standard error of the mean. *P<0.05 vs. Con; †P<0.05 vs. STZ-induced mice, n=6. Con, control; STZ, streptozotocin; TLR2A+STZ, Toll-like receptor 2 agonist, Pam,CysSK₄ and STZ.

Results

Metabolic parameters of Pam,CysSK₄-treated diabetic mice. Mice in the STZ group exhibited the typical manifestation of diabetes, including significant hyperglycemia, hyperlipidemia, weight loss and an increased kidney to body weight ratio compared with mice in the control group (Table I). Furthermore, mice in the TLR2A+STZ group exhibited a higher blood glucose level and lower body weight and serum albumin levels than mice in the STZ group (Table I).

Proteinuria, podocyte foot process production and renal function in Pam,CysSK₄-treated diabetic mice. As determined by measuring urine albumin levels over 24 h, compared with control mice, STZ-induced mice exhibited significant albuminuria (P<0.01), which was consistent with a previous report (15), and Pam,CysSK₄-treated diabetic mice had more severe proteinuria compared with untreated STZ-treated mice (P<0.05; Fig. 1A). Electron microscopy revealed podocyte foot process production in STZ-treated mice, with or without the TLR2 agonist Pam,CysSK₄ (Fig. 1B). From each image, the mean of the foot process width was calculated as described in the methods. It was demonstrated that Pam,CysSK₄, treatment exacerbated STZ-induced podocyte foot process production (P<0.05; Fig. 1C). Additionally, the renal function of Pam,CysSK₄-treated diabetic mice declined progressively, as reflected by the progressive increase in blood urea nitrogen and serum creatinine levels (Table I).

Renal histological examination. Histological sections of kidneys from the diabetic mice revealed glomeruli with signs of hypertrophy of the tuft and a mild expansion of the mesangial matrix (Fig. 2A). Glomerular fractional mesangial areas were significantly increased in Pam,CysSK₄-treated diabetic mice compared with untreated STZ-diabetic mice (P<0.05; Fig. 2B), and in STZ mice compared with controls. No histological changes in kidney were detected in the normal controls.

Expression of collagen IV and macrophage infiltration. Diabetes is associated with an increase in collagen IV protein expression in the kidneys (5,21). Collagen IV expression was elevated in Pam,CysSK₄-treated diabetic mice compared with the untreated STZ-diabetic mice (P<0.05;
Figure 1. Changes in the level of proteinuria and podocyte foot process production in STZ-induced and Pam3CysSK4-treated mice. (A) Proteinuria levels in the three groups. (B) Representative electron microscopy images of podocyte foot processes (red arrows) in the Con, STZ, and TLR2A+STZ groups (scale bar, 2 µm). (C) Quantification of foot process production. Data are presented as mean ± standard error of the mean. *P<0.01 vs. Con; †P<0.05 vs. STZ (n=6/group). STZ, streptozotocin; Con, control; TLR2A+STZ, toll-like receptor 2 agonist, Pam3CysSK4 and STZ; FPW, foot process width.

Figure 2. Histological examination of kidneys from STZ-induced and Pam3CysSK4-treated mice. (A) Representative renal histological changes (hematoxylin and eosin staining; red arrows) of Con, STZ-induced and TLR2A+STZ treated groups (scale bar, 50 µm). (B) Quantitative assessment of mesangial matrix fraction (%) in the kidneys of STZ-induced and Pam3CysSK4-treated mice. Data are presented as mean ± standard error of the mean. *P<0.01 vs. Con; †P<0.05 vs. STZ (n=6/group). STZ, streptozotocin; Con, control; TLR2A+STZ, toll-like receptor 2 agonist, Pam3CysSK4 and STZ.
Fig. 3. Immunohistochemical staining for collagen IV and F4/80 in the kidneys of STZ-induced and Pam3CysSK4-treated mice. (A) Representative immunostaining for collagen IV and F4/80 for the Con, STZ-induced and STZ+TLR2 treated mice. (B) Semi-quantification of collagen IV protein immunoreactivity in each group. (C) Semi-quantification of F4/80 protein immunoreactivity in the different groups. Data are presented as mean ± standard error of the mean.

**P<0.01 vs. Con; *P<0.05 vs. STZ (n=6/group).** STZ, streptozotocin; Con, control; TLR2A+STZ, toll-like receptor 2 agonist, Pam3CysSK4 and STZ.

Fig. 3A and B). F4/80-positive immunostaining was observed in the STZ-induced mouse kidney, predominantly in the renal interstitium. Additionally, there was a significant increase in F4/80 immunostaining in the kidneys of mice in the Pam3CysSK4 treatment group compared with the increase observed in the STZ-induced group (P<0.05; Fig. 3A and C).

Expression of TLR2 protein and mRNA in Pam3CysSK4-treated diabetic mouse kidneys. To investigate the effect of Pam3CysSK4 on the expression of TLR2 in the kidneys of diabetic mice, renal TLR2 protein and mRNA expression were examined by western blot analysis and RT-qPCR, respectively. Renal TLR2 protein expression was significantly upregulated in STZ-treated mice compared with control mice (P<0.05), and elevated further following Pam3CysSK4 treatment (P<0.05; Fig. 4A). Similarly, STZ-induced mice exhibited a significant increase in renal TLR2 mRNA expression compared with normal controls (P<0.05; Fig. 4B). Furthermore, the expression of TLR2 mRNA in the kidneys of Pam3CysSK4-treated mice was significantly elevated compared with the untreated STZ-induced mice (P<0.05; Fig. 4B).

Expression of MyD88 dependent signaling pathway in Pam3CysSK4-treated diabetic kidney. Western blot analysis results indicate there was significant upregulation of NF-κB p65 expression in STZ-treated mice compared with controls (P<0.05; Fig. 4C). The activation of NF-κB p65 was in accordance with the upregulation of MyD88 mRNA (Fig. 4B). In addition, the mRNA expression of MCP-1 was also markedly elevated in the diabetic kidney (Fig. 4B) and Pam3CysSK4-treated mice exhibited a significantly increased expression of MyD88 and MCP-1 mRNA (P<0.05; Fig. 4B), and activation of NF-κB p65, compared with the increase observed in STZ-induced mice (P<0.05; Fig. 4C).

Discussion

The current study evaluated the impact of a TLR2 agonist on renal injury in STZ-induced diabetic mice. The results
demonstrate that the TLR2 agonist increased urinary albumin excretion, podocyte foot process effacement, damaged renal function and aggravated glomeruli and renal tubular injury in the kidneys of diabetic mice. These events are associated with an increase in profibrotic and proinflammatory pathways.

Human TLRs, a subfamily of the pattern recognition receptors, are a large family consisting of 10 members (8). TLRs are divided into two groups according to their cellular localization. One group is expressed extensively on cell surfaces and includes TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11; another group consisting of TLR3, TLR7, TLR8 and TLR9, is expressed solely on intracellular vesicles, such as the endoplasmic reticulum, endosomes, lysosomes and endolysosomes (8). Different TLRs bind different ligands, attaching to specific adaptor proteins, thus triggering signaling cascades that lead to the activation of the inflammatory process. TLRs are primarily expressed on immune cells, including monocytes/macrophages, neutrophils, NK cells and dendritic cells (8,9). In addition, it has been demonstrated that non-immune cells, such as renal tubular cells, endothelial cells, podocytes and mesangial cells, express TLRs (9,10).

TLRs may be implicated in the pathogenesis and development of diabetes. It is widely considered that diabetes is an inflammatory disease (6,24) and TLRs are one of the primary mediators of the pathogenic processes of diabetes and its complications (25,26). It has previously been suggested that in STZ-induced diabetic rats, renal TLR2 expression is markedly increased, along with the expression of the proinflammatory genes MyD88 and CCL2 (15). Additionally, NK-κB activated infiltration of macrophages has been observed in STZ-induced diabetic rats (15). Furthermore, it was demonstrated that a genetic deficiency of TLR2 attenuated the increased inflammation and ameliorated renal dysfunction in STZ-induced diabetic mice (14). Thus, interventional studies are required to clarify the therapeutic role of TLR2 in the development of DN. There is currently no specific TLR2 inhibitor available, therefore TLR2 agonists are used to interfere with the TLR2 signaling pathway and modulate the development of diabetic renal injury. In the current study, the TLR2 agonist Pam3CysSK4 stimulated the TLR signaling pathway in diabetic mice and aggravated proteinuria, glomerular sclerosis and inflammatory infiltration. The results of the current study confirm that the TLR pathway may be associated with the development of DN.

It is widely accepted that structural changes, including alterations to the basement membrane, mesangial cell matrix and podocyte function, are responsible for proteinuria in DN. It has been indicated that the podocyte is the site of the initial injury in proteinuric renal diseases (22). In the present study, STZ-induced diabetic mice developed proteinuria with increased podocyte foot production, which was significantly exacerbated by Pam3CysSK4 treatment. This indicates that
Pam,CysSKK may accelerate diabetic renal injury by promoting podocyte dysfunction. A previous study demonstrated that overexpression of TLR2 in glomerular endothelial cells and podocytes induced cell apoptosis in acute kidney injury (27). This suggests that treatment with Pam,CysSKK may lead to increased podocyte apoptosis, resulting in significant proteinuria and podocyte foot process production compared with STZ treatment alone. It has been demonstrated that enhanced expression of collagen IV in the kidney accompanied increased mesangial cell proliferation in early diabetic nephropathy (5). The glomerular lesions observed in STZ-treated mice in the present study exhibited elevated collagen IV expression in the kidney, further confirming this association. Furthermore, Pam,CysSKK treatment exacerbated glomerular sclerosis and mesangial area expansion, and increased renal immunostaining for collagen IV was detected.

The results of the present study were consistent with previous studies that showed an association between upregulated expression of TLR2 in the kidneys of diabetic animals and an increase in inflammatory infiltration (14,15). Furthermore, the present study indicated that Pam,CysSKK may increase the inflammatory response and aggravate proteinuria and renal damage. A concomitant increase in the NF-κB p65 subunit with exposure to Pam,CysSKK was also detected. NF-κB is a nuclear transcription factor regulated by TLRs with a function to initiate transcription of proinflammatory genes (28,29). NF-κB, a key effector, has been implicated in the progression of DN and its activation leads to increased production of pro-inflammatory cytokines, cell cycle genes, profibrotic and extracellular matrix genes involved in DN (3,30). Thus, a TLR2 agonist may have an effect on the progress of DN through the TLR2/NF-κB pathway. Furthermore, an increase in the expression of proinflammatory genes, MyD88 and MCP-1 was observed in the current study. TLR2 signals primarily induce inflammatory responses through the MyD88-dependent pathway (14). Therefore, the results of the current study suggest that Pam,CysSKK activates the TLR2-MyD88-NF-κB pathway, leading to an increased production of chemokine MCP-1. Our data indicated that the mechanism initiated by Pam,CysSKK, which aggravates renal injury in diabetic mice, activates the TLR2-MyD88-NF-κB pathway and promotes an inflammatory response in the kidney. However, the indirect effect of blood glucose cannot be ignored as hyperglycemia may induce inflammatory cytokine secretion, activate inflammatory pathways and induce oxidative stress (7,24). In the current study, mice treated with Pam,CysSKK exhibited an elevated blood glucose level compared with the STZ-induced group. A previous study indicated that Pam,CysSKK increased the apoptosis of pancreatic islet β cells and promoted diabetes in C57BL/6 male mice (31). Thus, following Pam,CysSKK treatment, severe hyperglycemia may accelerate the development of DN.

In conclusion, the results of the present study demonstrated that the TLR2 agonist Pam,CysSKK activated the TLR2-MyD88-NF-κB pathway, increased albuminuria and accelerated glomerular sclerosis, tubular injury and inflammatory cell infiltration in diabetic mice. This indicates that enhanced renal expression of TLR2 is associated with inflammatory infiltration in DN, and that treatment with the TLR2 agonist Pam,CysSKK aggravates renal injury in diabetic mice.

Acknowledgements

The present study was supported by Natural Science Foundation of Guangdong Province (grant no. 2014A020212423), the Medical Scientific Research Foundation of Guangdong Province (grant no. B2014327), the Foundation of Shenzhen Basic Research Project (grant nos. JCYJ2015032414171629 and JCYJ20130401092802783) and the Fundamental Research Funds for the Shenzhen University (grant no. 201411).

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