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NLRP3 deficiency ameliorates renal inflammation and fibrosis in diabetic mice

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ABSTRACT

Diabetic nephropathy (DN) is the leading cause of end-stage renal disease. Activation of the nucleotide binding and oligomerization domain-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome has been reported in diabetic kidney, yet the potential role of NLRP3 inflammasome in DN is not well known. In this study, we explored the role of NLRP3 inflammasome on inflammation and fibrosis in diabetic kidney using NLRP3 knockout mice. Renal expression of NLRP3, caspase-1 p10, interleukin-18 (IL-18) and cleaved IL-1β was increased in diabetic wild-type (WT) mice at 24 weeks. NLRP3 knockout (KO) improved renal function, attenuated glomerular hypertrophy, glomerulosclerosis, mesangial expansion, interstitial fibrosis, inflammation and expression of TGF-β1 and connective tissue growth factor (CTGF), as well as the activation of Smad3 in kidneys of STZ-induced diabetic mice. In addition, NLRP3 KO inhibited expression of thioredoxin-interacting protein (TXNIP) and NADPH oxidase 4 (Nox4) and superoxide production in diabetic kidneys. The diabetes-induced increase in urinary level of 8-hydroxydeoxyguanosine (8-OHdG) was attenuated in NLRP3 KO mice. In vitro experiments, using HK-2 cells, revealed that high glucose (HG)-mediated expression of TXNIP and Nox4 was inhibited by transfection with NLRP3 shRNA plasmid or antioxidant tempol treatment. Silencing of the NLRP3 resulted in reduced generation of reactive oxygen species (ROS) in HK-2 cells under HG conditions. Furthermore, we also found exposure of IL-1β to HK-2 cells induced ROS generation and expression of TXNIP and Nox4. Taken together, inhibition of NLRP3 inflammasome activation inhibits renal inflammation and fibrosis at least in part via suppression of oxidative stress in diabetic nephropathy.

1. Introduction

Diabetic nephropathy (DN), one of the chronic kidney diseases, is the leading cause of end-stage renal disease (ESRD). The progression of DN is characterized by an increase in urinary albumin excretion, hypertension, glomerulosclerosis, and an eventual reduction in glomerular filtration rate, leading to ESRD. These changes are related to a remodeling of the renal structure, including glomerular and tubular hypertrophy, inflammation and extracellular matrix accumulation (Wada and Makino, 2013). Despite the available modern therapies of glycemic and blood pressure control, many patients continue to show progressive renal damage (Fineberg et al., 2013; Fernandez-Fernandez et al., 2014). Therefore, it is extremely important to identify novel interventions to halt the progression of DN.

Previous evidence suggests that chronic sterile inflammation plays an important role in pathogenesis and progression of DN (Navarro-Gonzalez and Mora-Fernandez, 2008). In recent years, it receives serious attention that diabetic renal fibrosis disease is a kind of a chronic inflammatory response (Navarro-Gonzalez et al., 2011). Diabetic nephropathy is accompanied by inflammatory responses in the kidney, characterized by up-regulation of inflammatory cytokines and infiltration of inflammatory cells. Macrophages and T cells accumulate in the glomeruli and interstitium in human DN, even in the early stages of the disease (Galkina and Ley, 2006). Kidney inflammation correlates strongly with the development of hyperglycaemia and glycated haemoglobin, and is driven by an increased kidney production of chemokines and proinflammatory cytokines in diabetic animals (Chow et al., 2004). Inhibition of inflammatory cell recruitment into the kidney has

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been shown to be protective in experimental diabetic kidney disease (Lim et al., 2009). Hence, the genetic or pharmacological approaches which reduce inflammation are potential therapeutic strategies for DN.

Accumulating evidence indicates that sterile inflammation is mediated through the inflammasome, a large cytosolic multiple protein complex, and regulates proinflammatory cytokine IL-1β and IL-18 production (Chen and Nunez, 2010; Davis et al., 2011). Nucleotide-binding oligomerization domain-like pyrin domain containing protein 3 (NLRP3) inflammasome is the best characterized inflammasome. The NLRP3 inflammasome recruits the apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and caspase-1, which results in caspase-1 activation to cleave its substrates including the precursors of inflammatory cytokine IL-1β and IL-18 to their bioactive form (Schroder et al., 2010). Previous studies have demonstrated that NLRP3 inflammasome played an important role in different renal damages. NLRP3−/− mice displayed less tubular injury, inflammation, and fibrosis after UUO, associated with a reduction in caspase-1 activation and maturation of IL-1β and IL-18 (Vilaysane et al., 2010). A specific inhibitor of the NLRP3 inflammasome, CP-456,773 could attenuate crystal-induced kidney fibrosis in mice (Ludwig-Portugall et al., 2016). Hyperuricemia-induced NLRP3 inflammasomes activation in macrophages contributed to the progression of DN (Kim et al., 2015). In addition, NLRP3 inflammasome activation has lately been reported in diabetic kidney, and NLRP3 or caspase-1 deficiency ameliorated albuminuria and the fractional mesangial area in diabetic mice (Shahzad et al., 2015). Moreover, inhibition of NLRP3 inflammasome activation may become a target for treating diabetic nephropathy (Wu et al., 2018). Although NLRP3 inflammasome activation is shown to initiate sterile inflammatory responses in DN, the role of NLRP3 inflammasome on diabetic kidney injuries has not been known clearly.

In the present study, we used NLRP3 knockout ($NLRP3^{-/-}$) mice with diabetes to investigate the roles of NLRP3 on renal function, extracellular matrix accumulation, fibrosis, inflammatory cell infiltration and oxidative stress. Meanwhile, we evaluated the effect of NLRP3 on TXNIP expression and ROS generation in HK-2 cells under high glucose conditions.

2. Materials and methods

2.1. Antibodies and other reagents

Streptozotocin (STZ), IL-1β, D-glucose and tempol were purchased from Sigma (St. Louis, MO, USA). Antibodies against NLRP3, caspase-1, caspase-1 p10 and fibronectin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Collagen I, TXNIP, Nox4, CTGF, monocyte chemoattractant protein-1 (MCP-1), F4/80 and IL-18 antibodies were obtained from Abcam (Cambridge, UK). Antibodies against IL-1β, *p*-Smad3 and Smad3 were purchased from Cell Signaling Technology (Beverly, MA, UAS). TGF-β1 and β-actin antibodies were obtained from Proteintech (Chicago, IL, USA). Collagen IV antibody and biochemical parameters reagent kits in urine and plasma were purchased from BioSino Bio-technology and Science Inc (Beijing, China). NLRP3 shRNA plasmid was obtained from Yingrun Biotechnology (Changsha, China). FuGENE-HD transfection reagent and the reverse transcription system were obtained from Promega (Madison, WI, USA). TRIzol reagent was obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). SYBR Premix Ex TaqTM II was purchased from Takara (Shiga, Japan).

2.2. Animals

C57BL/6J background $NLRP3^{-/-}$ (NLRP3 knockout, NKO) mice were generated by transcription activator-like effector nucleases (TALEN) technique. Male wild-type (WT) littermates were used as control. Mice were housed in the animal facilities of Hebei Medical University with 22 °C, a 12 h:12 h light-dark cycle and free access to food and water. Diabetes was induced by intraperitoneal injections of STZ (50 mg/kg in fresh 0.1 M sodium citrate buffer, pH 4.5) daily for 5 days, and control (nondiabetic) groups received citrate buffer. Three days after the injection, the mice with blood glucose greater than 16.7 mmol/l were regarded as successful models. The animals were culled at 24 weeks after the onset of diabetes. The kidneys were rapidly dissected, weighed and snap-frozen or processed in paraffin for further analysis. All animal studies were approved by the Animal Ethics Committee of Hebei Medical University.

2.3. Measurements of urine 8-hydroxydeoxyguanosine (8-OHdG)

Urine specimens were centrifuged at 1500 rpm for 10 min to remove particulates. The supernatants were used, and 8-OHdG levels were measured using a competitive *in vitro* enzyme-linked immunosorbent assay (ELISA) kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's descriptions.

2.4. Tissue histology and immunohistochemistry

Kidneys were fixed in 4% paraformaldehyde overnight, and embedded in paraffin. Two- and 4-μm sections were prepared. Two-micrometer sections were stained with periodic acid-Schiff (PAS) and Masson's trichrome staining. A semiquantitative index was used to evaluate the degree of glomerular mesangial expansion and sclerosis (Zhao et al., 2006). Briefly, each glomerulus on a single section was graded from 0 to 4, with 0 representing no lesion, and 1, 2, 3, and 4 representing mesangial matrix expansion or sclerosis, involving ≤25, 25 to 50, 50 to 75, or > 75% of the glomerular tuft area, respectively. Immunohistochemistry for antibodies on renal sections was performed with SP kit according to the instruction. Paraffin-embedded tissue sections were deparaffinised in xylene and rehydrated through graded ethanol. Internal peroxidase was inactivated with 3% hydrogen peroxide in 100% methanol for 30 min. Antigen retrieval was subsequently performed by autoclaving for 15 min at 121 °C in sodium citrate buffer (pH 6.0). After blocking with 10% normal goat serum for 30 min at room temperature, the sections were incubated with primary antibodies for fibronectin, collagen IV, TXNIP, Nox4, TGF-β1, CTGF, MCP-1 and F4/80 overnight at 4 °C. Sections were then washed and incubated with biotinylated secondary antibody and horseradish peroxidase-conjugated streptavidin. Labeling was visualized with 3,3-diaminobenzidine to produce a brown color, and sections were counterstained with hematoxylin. Staining was analyzed under light microscopy by two independent, blinded observers. The collected images were assessed by National Institutes of Health ImageJ software (Lo et al., 2012; Shi et al., 2013).

2.5. Immunofluorescence assay

The expression of collagen I was detected by immunofluorescence assay. The renal tissues were fixed with 4% paraformaldehyde at room temperature for 10 min and permeabilized in 0.2% Triton X-100 for 10 min. After 30 min goat serum blocking at 37 °C, the renal tissues were incubated with antibody against collagen I in PBS overnight at 4 °C. Then, the renal tissues were incubated with FITC-labeled secondary antibody for 1 h at 37 °C. Washed five times with PBS, cell nuclei were then counterstained with DAPI dissolved in PBS (10 g/ml) for 10 min at room temperature. The sections were read under a fluorescence microscope (Olympus, Japan). The collected images were assessed by National Institutes of Health ImageJ software (Lo et al., 2012; Shi et al., 2013).

2.6. Cell culture

HK-2 cells (ATCC, American Type Culture Collection, Manassas, VA)