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The mTOR promotes oxidative stress-induced apoptosis of mesangial cells in diabetic nephropathy

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ABSTRACT

Glomerular mesangial cell (MC) apoptosis is one of the important mechanisms of glomerulosclerosis, which induces an increased severity of albuminuria and promotes the development of diabetic nephropathy (DN). However, the mechanism by which high glucose (HG) induces MCs apoptosis is not fully understood. In the present study, we investigated the effects of mTOR signalling on apoptosis in cultured MCs exposed to HG and in type I diabetes, and tried to clarify the specific mechanisms underlying these effects. *In vitro*, exposure of MCs to HG stimulated ROS production, decreased the antioxidant enzyme superoxide dismutase (SOD) activity and glutathione (GSH) level, increased nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity, upregulated P53 expression and Bax/Bcl-2 ratio and enhanced cleavage of caspase 3, resulting in an increase in programmed cell death. Pretreatment of the cells with rapamycin ameliorated oxidative stress, reduced the number of apoptotic cells induced by HG and caused the downstream effects of mTOR activation. *In vivo*, compared with control rats, diabetic rats had more apoptotic cells in glomeruli. Induction of diabetes increased the level of MDA and NADPH oxidase activity, decreased the SOD activity and GSH level, elevated the Bax/Bcl ratio and P53 expression and activated caspase 3. mTOR inhibitor rapamycin treatment prevented these changes further alleviated albuminuria and improved renal function. Taken together, our data suggest that mTOR plays a key role in mediating ROS-induced MC apoptosis in diabetic nephropathy, and these effects have been associated with the promotion of ROS production by upregulating the antioxidant enzyme and downregulating the NADPH oxidase activity.

1. Introduction

Diabetic nephropathy (DN) is one of the most serious microvascular complications of diabetes mellitus and leads to end-stage renal disease (ESRD). Glomerular mesangial cells (MCs) are known to play an important role in maintaining the structural integrity of the glomerular microvascular bed, providing mesangial matrix homeostasis and modulating glomerular filtration (Mishra et al., 2005). Emerging evidence has suggested that MC apoptosis increases the severity of albuminuria and contributes to diabetic glomerulosclerosis, which is involved in the pathogenesis and progression of DN (Hornigold et al., 2013; Yao et al., 2015). Thus, a better understanding of the underlying mechanisms of MC apoptosis is essential to illustrate the pathogenic mechanisms of DN.

Reactive oxygen species (ROS) are recognised as important mediators for several biologic responses, including proliferation, extracellular matrix deposition, and apoptosis (Maiese, 2015). Numerous data have indicated that ROS play an important role in the pathogenesis of DN (Matough et al., 2012; Giacco and Brownlee, 2010). Our previous studies also confirmed that the levels of ROS were increased in MCs in high-glucose concentrations and in the kidney cortex of *db/db* mice (Lu et al., 2013). In addition, both *in vivo* and *in vitro* studies have shown that ROS induce mesangial cell dysfunction and lead to apoptosis, which disturbs glomerular homeostasis and is involved in the pathogenesis of DN (Tavafi, 2013; Shah et al., 2013). However, the exact mechanism of hyperglycaemia-mediated oxidative stress in the procession of MC

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apoptosis is completely unknown.

Mammalian target of rapamycin (mTOR), a highly conserved nutrient-responsive regulator of cell growth found in eukaryotes, is a serine/threonine protein kinase existing in two complexes, mTORC1 or mTORC2, consisting of distinct sets of protein-binding partners (Saxton and Sabatini, 2017; Yang et al., 2013). mTORC1 is sensitive to rapamycin and is thought to regulate its downstream effects through p70 ribosomal protein S6 kinase (p70S6K) (Ma and Blenis, 2009). The abnormal activation of mTOR/p70S6K signalling has been implicated in the pathogenesis of DN, including glomerular hypertrophy, podocytefunction and renal fibrosis (Inoki et al., 2011; Kajiwara and Masuda, 2016). Recent experimental evidence has suggested that activation of mTOR increases the production of ROS, whereas inhibition of mTOR decreases its level (Kim et al., 2005). Tuñón et al. confirmed that the mTOR inhibitor rapamycin decreased the intracellular generation of ROS and inhibited NO production in rat hepatocytes, which was associated with NF-κB activation (Tuñón et al., 2003). Previous studies showed that mTOR is also involved in the phosphorylation/inactivation of Bcl-2 in microtubules treated with apoptotic agents. Velagapudi et al. demonstrated that exposure of proximal tubular elentary (PTE) cells to HG increased phosphorylation of mTOR, phosphorylation of Bcl-2 and caspase-3 activity, and pre-treatment of the cells with the mTOR inhibitor rapamycin reduced the number of apoptotic cells induced by HG (Velagapudi et al., 2011). Although it has been well established that mTOR is a critical factor in DN, the interrelationships between mTOR, oxidative stress and MC apoptosis have not been fully demonstrated *in vitro* or *in vivo*.

In the present study, we investigated the potential role of the mTOR pathway in oxidative stress and in promoting an apoptotic signalling cascade in HG-induced MCs and in a rat model of type 1 diabetes, to illuminate the possible mechanisms underlying these effects. The above descriptions provide evidence that mTOR may be a fundamental novel target for apoptotic MCs in DN.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (Certificate No.) weighing between 200 and 220 g were obtained from the Laboratory Animal Centre of Xuzhou Medical University (Xuzhou, China), following the Guiding Principles for Care and Use of Laboratory Animals of Xuzhou Medical University. The rats were divided into three groups of six rats per group. Group I (control): the rats were treated with an equivalent amount of sodium citrate buffer (0.1 moL/L, pH 4.5) by intraperitoneal injection. Group II (diabetes): the rats were injected intraperitoneally with 60 mg/kg body weight STZ (Sigma, St. Louis, MO, USA) dissolved in sodium citrate buffer as in group I to induce type 1 diabetes and were treated with 1% carboxyl methyl cellulose (CMC) solution. Group III (diabetes + rapamycin): the rats were injected with streptozocin (STZ) as in group II, and were intragastrically treated with 1 mg/kg rapamycin (Sirolimus, Sigma, St. Louis, MO, USA) dissolved in 1% CMC solution daily. The induction of the diabetic state was confirmed by the fasting blood glucose (FBG) level at 72 h after STZ administration. The rats with FBG levels above 13.9 mmoL/L were considered diabetic. After 14 weeks, the rats were placed in metabolic cages for urine collection, and the blood samples were also collected. Animals were sacrificed, and the kidneys were removed rapidly. The kidneys were stored at −80°Cfor biochemical analysis.

2.2. Cell culture

The rat mesangial cell line HBZF-1, purchased from the China Centre for Type Culture Collection (Wuhan, China), were grown in Dulbecco's Modified Eagle's Medium (MEM) containing 100 units/ml penicillin, 100 μg/ml streptomycin, 10% foetal bovine serum (FBS) and 5.56 mmoL/L glucose. Confluent cells were grown in serum-free Dulbecco's MEM media for 24 h before the experiments. Then, the cells were cultured with 30 mmoL/L glucose (high glucose, HG) for 12, 24 and 48 h.

2.3. Measurement of renal function and biochemical parameters

Blood glucose was measured by test strips (Johnson, CA, USA). The values of urine protein were tested by the urine protein quantification kit (Jiancheng Bioengineering Institute, Nanjing, China), creatinine (Cr) and blood urea nitrogen (BUN) were determined using the Cr and BUN assay kits (Huili Biotech, Changchun, China).

2.4. Detection of intracellular ROS

Changes in intracellular ROS levels were determined by measuring the oxidative conversion of cell permeable 2′,7′-dichlorofluorescein diacetate (DCFH-DA) to fluorescent dichlorofluorescein (DCF) using an ROS detection kit (Beyotime Institute of Biotechnology, Nantong, China). Cells were grown in 6-well plates and serum-starved for 24 h. Cells were then washed with serum-free Dulbecco's MEM media and then incubated with DCFH-DA at 37°Cfor 20 min before the experiment. Then, the DCF fluorescence distribution of 10,000 cells was detected at excitation and emission wavelengths of 488 and 525 nm, respectively, by flow cytometry (Miltenyi Biotec, Bergisch Gladbach, Germany).

2.5. Western blotting analysis

Homogenates from glomeruli isolated from the renal cortex were prepared in lysates (50 mmoL/L Tris HCl, pH 7.6, 150 mmoL/L NaCl, 1 mmoL/L EDTA, 1% NP-40, 1 mmoL/L PMSF, 1 mmoL/L Na_3VO_4 and 20 mmoL/L NaF). Homogenates were incubated for 30 min at 4 °C and centrifuged at 12,000 g for 30 min at 4 °C. HBZF-1 cells were lysed in the lysis buffer described above and incubated at 4 °C for 30 min. Then, the lysates were centrifuged at 12,000 g for 30 min at 4 °C. Protein concentrations were determined with a BCA Protein Assay Kit (Thermo Scientific-Pierce, MA, USA) following the manufacturer's protocol. For immunobloting, proteins (40–80 μg) were separated on 8% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Then, the membrane was blocked in phosphate-buffered saline (PBS) containing 5% bovine serum albumin (BSA) for 1 h at room temperature, and incubated with primary antibodies overnight at 4 °C. mTOR, phosphate-mTOR, p70S6K, phosphate-p70S6K antibodies were purchased from Cell Signalling (Beverly, MA, USA). P53, Bcl-2, Bax, cleaved-caspase 3 and β-actin antibodies were purchased from Bioworld Technology (St. Louis, USA). NOX4, NOX2 antibody were obtained from Abcam (Cambridge, UK). The primary antibodies were detected using alkaline phosphatase-conjugated immunoglobulin G (IgG; Bioworld Technology, St. Louis, USA) at room temperature for 1 h. Membranes were developed colourimetrically by BCIP/NBT alkaline phosphatase colour development kit (Beyotime Institute of Biotechnology, Nantong, China). The density of the bands was quantified by densitometric analysis using National Institutes of Health ImageJ software.

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