Molecular and Cellular Endocrinology 405 (2015) 74–83

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Glycated albumin triggers fibrosis and apoptosis via an NADPH oxidase/Nox4-MAPK pathway-dependent mechanism in renal proximal tubular cells

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ARTICLE INFO

Article history:
Received 3 October 2014
Received in revised form 5 February 2015
Accepted 6 February 2015
Available online 11 February 2015

Keywords:
GA
Tubulointerstitial fibrosis
Apoptosis
MAPK pathway
NADPH oxidase
Nox4

ABSTRACT

Glycated albumin (GA), an Amadori product used as a marker of hyperglycemia and the early-stage glycation products compared to AGEs, might further promote kidney lesions in diabetic nephropathy (DN). However, the mechanisms how GA cause proximal tubular cells damage remain poorly understood. In this study, we investigated the effects of GA on fibrosis and apoptosis of renal proximal tubular cells (NRK-52E) *in vitro* experiments. Our results showed that GA promoted α-SMA, fibronectin (FN) and TGF-β expressions in NRK-52E cells. GA also increased cell apoptosis and stimulated the expressions of pro-caspase 3/cleaved-caspase 3. GA overloading enhanced the phosphorylation of MAPK pathway. GA-induced α-SMA, FN, TGF-β and caspase 3 expressions were completely suppressed by the NADPH oxidase inhibitor apocynin (Apo), the reactive oxygen species (ROS) scavenger *N*-acetylcysteine (NAC) and the latent antioxidant Astragaloside IV (AS-IV). Real-time PCR showed that GA increased Nox1, Nox2 and Nox4 mRNA expressions, especially the Nox4 expression. Furthermore, Nox4 siRNA blocked GA-induced tubular damages and the MAPK pathway activation. These results demonstrate that GA increases the permissiveness of proximal tubular cells to fibrosis and apoptosis *in vitro* by triggering a pathway that involves NADPH oxidase/Nox4-MAPK signaling pathway. This event may represent a key cellular effect in increasing the susceptibility of tubular cells to fibrosis and apoptosis when the tubules cope with a high GA load. This effect is instrumental to renal damage and disease progression in patients with DN.

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1. Introduction

Diabetic nephropathy (DN) is a major microvascular complication of diabetes mellitus and the main cause of end-stage renal diseases. The enhanced glomerular and tubulointerstitial accumulation of extracellular matrix (ECM), apoptosis of renal tubular epithelial cells and proliferation of interstitial mesenchymal cells are hallmark features of DN (Verzola et al., 2007). Although DN is considered as a glomerular disease, recent studies suggested that proximal tubular changes were evident even in the early stage (Hasegawa et al., 2013). So far, the pathological mechanisms of DN remain unclear but increasing evidence demonstrated that oxidative stress (OSS) participated in the pathogenesis of DN (Rösen et al., 2001; Sedeek et al., 2010; Shah et al., 2007; Stanton, 2011; Xu et al., 2012). The excessive production of reactive oxygen species (ROS) caused by many factors in OSS can directly disturb the redox balance and reduce the amount of antioxidant enzymes (Banerjee and Vats, 2013). It also promotes the generation of various cytokines and stimulates the activation of signaling pathways to affect the bioactivity of renal cells which could ultimately initiate and participate in the development of DN (Rösen et al., 2001; Stanton, 2011). Of the many enzymatic systems in ROS generation in the kidney, NADPH oxidase appears to be particularly important (Barnes and Gorin, 2011). Thus, suppressing the generation of ROS induced by NADPH oxidase activation in OSS has important significance in the prevention and treatment of DN.

It is well-known that the non-enzymatic glycation products obviously increase in diabetic patients compared to non-diabetic subjects, and these glycated proteins have been suggested to contribute to the onset and progression of diabetic complications (Raghav and Ahmad, 2014). Glycated albumin (GA) is an Amadori product formed through the non-enzymatical condensation reaction of glucose with reactive albumin. Amadori products undergo further irreversible reactions to yield AGEs (Furusyo and Hayashi,

Abbreviations: GA, glycated albumin; DN, diabetic nephropathy; ROS, reactive oxygen species; OSS, oxidative stress; ECM, extracellular matrix; Apo-, apocynin; FN, fibronectin; AS-IV, Astragaloside IV.

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http://dx.doi.org/10.1016/j.mce.2015.02.007
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2013). *In vivo*, GA increases with the presence of hyperglycemia and reflects the fluctuation of glucose level over the past 2–3 weeks (Kim and Lee, 2012). Previous studies found that GA was not only a biomarker but also a damage factor. For example, GA could associate with the abnormal renal nephrin and VEGF expression to promote proteinuria and glomerulosclerosis in diabetes (Cohen et al., 2005), suppress insulin secretion by impairing glucose metabolism in rat pancreatic β-cells (Shiraki et al., 2011) and stimulate cultured retinal microglial to secrete inflammatory cytokines (Ibrahim et al., 2011). Most recently, we also demonstrated that GA induced epithelial-to-mesenchymal transition in proximal tubular cells by increasing ROS generation (Qi et al., 2014). Many reports have indicated that MAPK pathway played an important role in the pathogenesis of DN (Dronavalli et al., 2008; Hattori et al., 2001). Furthermore, intracellular ROS production may inhibit tyrosine phosphatases, which would lead to the phosphorylation of MAPK pathway (Saldanha et al., 2013). However, the roles of the MAPK pathway in the process of GA-induced fibrosis and apoptosis of proximal tubules have not yet been clearly described.

Given the earlier note, the current work aimed to determine whether the inhibition of ROS generation or NADPH oxidase activation could reverse GA-induced proximal tubular fibrosis and apoptosis, and explore the mechanism underlying OSS-mediated injury in DN with special focus on the MAPK pathway.

2. Materials and methods

2.1. Materials

GA, 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA), *N*-acetylcysteine (NAC), and apocynin (Apo) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The MAPK pathway inhibitors SB203580 (SB), SP600125 (SP) and PD98059 (PD) were bought from Enzo Life Sciences (New York, NY, USA). Astragaloside IV (AS-IV) (Ronghe Co., Shanghai, China) was dissolved in DMSO at 10 mg/ml to create a stock solution. Primary antibodies against α-SMA, pP38, pERK1/2 (Epitomics, Burlingame, CA, USA); GAPDH, pJNK, procaspase 3/cleaved caspase 3 (Cell Signaling Technology, Beverly, MA, USA); TGF-β, FN (Immunoway, Newark, DE, USA) were purchased from commercial sources. The FN ELISA assay kit was gained from Abcam (Cambridge, UK). The TGF-β ELISA assay kit was obtained from R&D Co. (Minneapolis, MN, USA). TUNEL assay kit was bought from Roche Diagnostics (Mannheim, Germany). Small interfering RNA (siRNA) specific for rat Nox4 was obtained from Shanghai GenePharma Co., Ltd.

2.2. Cell culture

NRK-52E, an immortalized proximal tubular epithelial cell line from normal rat kidneys, was obtained from the Institute of Biochemistry and Cell Biology (Shanghai, China). The cells were grown in DMEM (5.5 mM glucose) supplemented with 5% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were maintained in an atmosphere of 5% CO_2, and 95% air at 37 °C. The medium was changed every 3 days until the cells were confluent. Approximately 70–80% confluent NRK-52E cells were incubated with serum-free medium overnight to arrest and synchronize the cell growth. The cells were treated with GA at a final concentration of 400 μg/ml for 24 h (Qi et al., 2014). In experiments using inhibitors or antioxidants, cells were preprocessed for 30 min with 20 μM of the NADPH oxidase inhibitor Apo, 10 μM of the P38 inhibitor SB203580 (SB), 10 μM of the JNK inhibitor SP600125 (SP), 10 μM of the ERK1/2 inhibitor PD98059 (PD), 5 μM of the classic antioxidant NAC or 10 μM of the latent antioxidant AS-IV before treatment with exogenous GA.

2.3. In situ *cell death detection by TUNEL assay*

TUNEL staining was conducted using a commercial kit (*in situ* cell death detection kit) according to the manufacturer's instruction. In brief, NRK-52E cells were seeded in 6-well plates and then cultured under GA stimulation for 24 h. Notably, cells in treatment groups should be pretreated with NAC or AS-IV as the antioxidants for 30 min. After incubation, the cells were fixed with 4% paraformaldehyde at room temperature for 1 h, permeabilized for 2 min on ice, and then washed cells and incubated with TUNEL reaction mixture in the dark for 1 h at 37 °C. At the end of the experiments, photos were taken by fluorescence microscopy immediately.

2.4. Nox4 siRNA transfection

NRK-52E cells were plated on 6 cm^2 trays prior to transfection and allowed to reach about 50% confluence. On the day of transfection, the appropriate cells were washed with PBS and cultured with serum- and antibiotic-free DMEM medium. Negative siRNA or Nox4 siRNA was transfected into these cells using the Lipofectamine 2000 transfection reagent (Invitrogen, USA) according to the manufacturer's protocol. After transfection for 6 h, the cells were washed with PBS for 2 times, and fresh DMEM containing 5% FBS without antibiotics was added to the cells. The cells were then further incubated at 37 °C in 5% CO_2. After 24 h, the cells were stimulated according to the experiment requirements. Another 24 h later, the cells were collected for the following uses.

2.5. Quantitative real time reverse transcription polymerase chain reaction (qRT-PCR)

The total RNA was isolated using the AxyPrep™ Multisource Total RNA Miniprep kit (Axygen, USA). An equivalent amount of RNA was converted into complementary DNA (cDNA) with the PrimeScript™ RT reagent Kit (Takara, Japan). Subsequently, qRT-PCR was performed using an ABI7500 Sequencing Detection System and SYBR® Premix ExTaq™ (Takara, Japan). All of the procedures were performed according to the manufacturer's protocols. Cycling condition was as follows: 40 cycles at 95 °C for 5 s and 60 °C for 34 s. The comparative $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression level of each target gene, with GAPDH serving as the housekeeping gene. qRT-PCR was performed to analyze the mRNA expressions of α-SMA, FN, TGF-β, Nox1, Nox2 and Nox4 in NRK-52E cells. All the oligonucleotide primers were designed by Shanghai Sangni Company (Table 1).

2.6. Measurement of caspase 3 activity

The activation of caspase 3 was measured using a commercially available kit (Applygen Technologies Inc., China). NRK-52E cells were seeded in 6-well plates and added GA to stimulation for 24 h with or without the pretreatment of NAC, AS-IV, Apo, SB, SP or PD for 30 min. After incubation, the cells were lysed in cell lysis buffer

Table 1
Real-time reverse transcription-polymerase chain reaction (RT-PCR) primers.

Gene	Upstream sequence (5′–3′)	Downstream sequence (5′–3′)
α-SMA	TGTGCTGGACTCTGGAGATG	GAAGGAATAGCCACGCTCAG
FN	CAACAATTCCTGGCGTTACCT	AAGCCCTGTATTCCGTCTCCTT
TGF-β	CAACAATTCCTGGCGTTACCTT	AAGCCCTGTATTCCGTCTCCTT
Nox1	TTCTGGGAAACCTGCCTTTAG	ACAGGTGGGAGGGAAGATTA
Nox2	ACCCTCCTATGACTTGGAAAG	TGATGACCACCTTCTGTTGAG
Nox4	TTCTGGACCTTTGTGCCTATAC	CCATGACATCTGAGGGATGATT
GAPDH	GCAAGTTCAATGGCACAG	GCCAGTAGACTCCACGACAT

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