Biochemical and Biophysical Research Communications xxx (2018) 1–8

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

RhoA protects the podocytes against high glucose-induced apoptosis through YAP and plays critical role in diabetic nephropathy

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ARTICLE INFO

Article history:
Received 20 August 2018
Accepted 31 August 2018
Available online xxx

Keywords:
RhoA
YAP
Podocyte apoptosis
Diabetic nephropathy

ABSTRACT

Background: Podocyte apoptosis is important mechanism that leading to proteinuria in Diabetic nephropathy (DN), but the underling mechanisms that cause podocyte apoptosis in DN are not very clear. We have recently demonstrated that RhoA, a small GTPase protein, effectively protected podocyte apoptosis induced by LPS and ADR in vitro. However, the potential role of RhoA in DN is unknown.
Methods and results: Conditionally immortalized mouse podocyte cells, C57BL/KsJ, *db/db* diabetic mice, and renal biopsies from patients with DN were used for study. The treatment of podocytes with high glucose (HG) for 48h significantly induced cell apoptosis and decreased RhoA expression and its activity. The expression of RhoA was also decreased in glomerular podocytes of db/db mice and patients with DN. Knockdown of RhoA by siRNA contributed in the apoptosis of podocyte and induced proteinuria in db/db mice. Beyond the increased pro-apoptotic Bax and the decreased anti-apoptotic Bcl-2, RhoA knockdown also inhibited the expression of a nuclear protein of YAP in podocyte. Over expression active form of YAP completely abolished the apoptosis of podocyte induced by RhoA knockdown.
Conclusion: RhoA plays a critical role in DN probably by mediating the podocyte apoptosis through YAP. RhoA may be a novel molecular target for the treatment of DN.

1. Introduction

Diabetic nephropathy (DN) is a common complication of diabetes. Although DN is characterized by glomerular damage and proteinuria, the underlying mechanisms about DN pathogenesis are still not fully understood. Podocytes are highly differentiated cells and play an important role in maintaining the integrity of glomerular filtration barrier [1–3]. The loss of podocytes will inevitably disturb the function of glomerular and sequentially causes proteinuria. More and more evidences have showed that podocyte apoptosis is one of the most important mechanisms that leading to DN [4–6]. Therefore, preventing podocyte apoptosis will be a promising therapeutic target for DN. Nevertheless, the underlying mechanisms leading to podocyte apoptosis in DN are still not very clear.

Previous studies have reported that proteinuria are associated with remarkable cytoskeletal injury and foot process effacement [7,8]. Ras homolog gene family, member A (RhoA), one of the most well studied Rho GTPases has been demonstrated to play a key role in maintaining the function of cytoskeletal architecture [9,10]. Active form of RhoA binds to its downstream effector mammalian diaphanous-related formins (mDias), which relieves mDias' autoinhibition of the DID/DAD interaction and promotes stress fibers formation [11,12]. The deficiency of RhoA in podocytes can lead to the loss of stress fibers and induce proteinuria [13,14]. It has demonstrated that RhoA deficiency leaded to the apoptosis of A549 and HepG2 cells [7,15], by inhibiting Yes-associated protein (YAP) [16], a key downstream cascade of the Hippo pathway for cell survival and proliferation [17–21]. Our previous study also indicated that RhoA deficiency could reduce stress fibers formation and induce podocyte apoptosis by inhibiting YAP in vitro [8].

YAP is a key downstream cascade of the Hippo pathway for cell

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https://doi.org/10.1016/j.bbrc.2018.08.204
0006-291X/

survival and proliferation [17–21]. In normal conditions, dephosphorylated form of YAP is located at the nucleus and functions as a transcriptional co-activator to interact with TEA domain family member transcriptional factors thereby induce target gene expression [22]. Phosphorylated form of YAP is sequestrated in the cytoplasm and maintains inactive [23,24]. Insufficient stress fibers induced by loss of RhoA can decrease transcriptional activity and nuclear localization of YAP [16,25,26]. Our previous study has indicated that loss of RhoA in LPS or ADR-injured podocytes could induce apoptosis by inhibiting YAP [8]. However, the role of RhoA in HG-injured podocytes, db/db mice and DN patient is unknown.

Using conditionally immortalized mouse podocyte cells, diabetic mice, and renal biopsies from patients with DN for experiments, this study is aimed to investigate the potential role of RhoA in DN, and further to explore the relevant mechanism which may be one potent way to prevent podocyte apoptosis and proteinura in DN.

2. Methods

2.1. Immortalized mouse podocyte cells, diabetic mice, and renal biopsies from patients with DN

The conditionally immortalized mouse podocyte cell line (MPC) was given by Dr.Jochen Reiser (Rush University Medical Center, Chicago, IL, USA). The method of podocyte culture was described previously [27,28].

6 Male C57BL/KsJ *db/db* mice (8-week-old) and 6 age-matched wild-type mice were purchased from Model Animal Research Center of Nanjing University, 36 Male BALB/c mice (8-week-old) were from Center of Laboratory Animal Science of Guangdong. The db/db mice are obese and known to develop diabetic kidney disease. We collected urine once every two weeks in a metabolic cage for 24 h from week 12 to week 20. The entire animal experiment was performed according to the ARRIVE guidelines [29], and was approved by the Ethics Committee for animal research of the First Affiliated Hospital of Guangzhou Medical University. We anaesthetized mice by ketamine (70 mg/kg, i.p.) before killed, and then collected kidney tissues. Mouse albumin-specific ELISA kit (Bethyl Laboratories, Inc, USA) and creatinine kit (Cayman Chemical, USA) were used to measured mice urinary albumin and creatinine respectively according to the manufacturer's instructions. Proteinuria was expressed as the ratio of albumin to creatinine. Before we obtained renal biopsies tissues from patients with DN and renal cell carcinoma (adjacent normal tissues), written informed consents were given. Study of patients was approved by the Ethics Committee for human research of the First Affiliated Hospital of Guangzhou Medical University.

2.2. Transfection of siRNAs and plasmids

The small interfering RNAs (siRNAs) of control, RhoA and YAP were synthesized by RIBOBIO CO. LTD. (Guangzhou, China). siRNAs was transfected as described [28]. The sequences of siRNAs were as follows: RhoA-siRNA 5′-GGUAAGACAUGCUUGCUCA dTdT-3′, YAP-siRNA 5′-CGAGAUGA GAGCACAGACA dTdT-3′. 10^6 cells were treated with high glucose (HG, 30 mM) or siRNAs (50 nM) for 48 h.

As our previous study described [28], lentiviral packaging wild-type YAP or YAP S112A mutation plasmids (CS-Mm06093-Lv128, GeneCopoeia, China) were produced in HEK293T cells using the Lenti-Pac™ HIV Expression Packing Kit (HPK-LvTR-20, GeneCopoeia, China). Then podocytes were infected with the lentivirus particles. Cells under different conditions were collected 48h after transfection.

2.3. Real-time quantitative-PCR

The details of RT-PCR have been described in our previous study [28]. The primers related in this study are listed as follows: Bax, forward 5′-CTGGACCATAGGTCGGAGTG-3′, reverse 5′-AATTCGCCG GAGACACTCG-3'; Bcl-2, forward 5′-GTCGCTACCGTCGTGACTTC-3′, reverse 5′-CAGACATGCACCTACCCAGC-3'; GAPDH, forward 5′-AGGT CGGTGTGAACGGA TTTG-3′, reverse 5′-TGTAGACCAT GTAGTTGAG GTCA-3'.

2.4. Western blotting

The details of WB have been described [4,28]. The primary antibodies were as follows: rabbit anti-RhoA (Santa Cruz, USA, 1:500), rabbit anti-GAPDH (Bioworld Technology, China, 1:10000), rabbit anti-Histone(Cell Signaling Technology, USA, 1:3000), rabbit anti-YAP(Cell Signaling Technology, USA, 1:1000), rabbit anti-Bcl-2 (Santa Cruz, USA, 1:500), rabbit anti-Bax (Santa Cruz, USA, 1:500). Protein expression was quantified as the ratio of specific band to GAPDH or Histone (nuclear fractions).

2.5. Annexin V and propidium iodide staining assay

As described previously [28], cell apoptosis in different groups were detected by Annexin V/PI apoptosis detection kit according to manufacturer's protocol (Nanjing KeyGEN Biotech, China).

2.6. RhoA GTPase activation assay

As described previously [28], G-LISA RhoA Activation Assay Biochem kit (colorimetric assay, Cytoskeleton, USA) was used to detect active RhoA according to the manufacturer's instruction.

2.7. Murine models of RhoA knockdown

As described previously [30], five male BALB/c mice were received daily intravenous injections of control siRNA or RhoA-siRNA (siCon or siRhoA) for seven days respectively. Briefly, synthetic control or RhoA-specific siRNA (50 μg of siRNA dissolved in 1 ml of saline) was rapidly injected via tail vein into mice within 10 s, once a day for 7 consecutive days. Five blank control mice were intravenously injected with1 ml of saline. Urine of all mice was collected at day 0, 4, 7 before killed.

2.8. Podocyte number counting

The podocyte number per glomerulus was determined as described previously [31,32]. Rabbit polyclonal antibody Wilm's tumour antigen 1 (WT-1; sc-192, Santa Cruz, USA) was used to marked podocytes. The podocyte number per glomerulus is regarded as the average number of brown-stained nuclei from 20 randomly selected glomeruli.

2.9. Immunofluorescent staining and TUNEL staining

The procedure of immunofluorescent staining and TUNEL staining are described in our previous study [28]. The primary antibodies were as follow: goat anti-synaptopodin (Santa Cruz, USA, 1:100), goat anti-WT-1(Santa Cruz, USA, 1:100), rabbit anti-RhoA (Santa Cruz, USA, 1:100), rabbit anti-YAP (Santa Cruz, USA, 1:100). Culture podocytes were stained with phalloidin (Cytoskeleton, USA, 1:500) and DAPI (Sigma, St Louis, MO). Cell apoptosis in kidney sections was detected by TUNEL kit (Roche Molecular Biochemicals, Mannheim, Germany) as described in our previous study [28]. Cells both positive for TUNEL and WT-1 are apoptotic

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