Biochemical and Biophysical Research Communications xxx (2018) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

miR-423-5p suppresses high-glucose-induced podocyte injury by targeting Nox4

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ARTICLE INFO

Article history:
Received 3 September 2018
Accepted 11 September 2018
Available online xxx

Keywords:
Podocyte injury
miR-423-5p
Nox4
ROS
Apoptosis
p38 MAPK

ABSTRACT

Podocyte injury plays crucial roles in the pathogenesis of diabetic nephropathy (DN). Aberrant microRNAs (miRNAs) have been suggested to contribute to podocyte injury. However, whether miR-423-5p could alleviate high glucose (HG)-mediated podocyte injury and the underlying mechanisms remains unclear. In this study, we found that patients with DN have reduced miR-423-5p and elevated Nicotinamide adenine dinucleotide phosphate oxidase 4 (Nox4) expressions in clinical renal tissues, and HG induced Nox4 but suppressed miR-423-5p expressions in cultured podocytes in a time-dependent manner. Moreover, overexpression of miR-423-5p antagonized HG-stimulated podocyte injury by enhancing cell viability, inhibiting reactive oxygen species (ROS) production, suppressing cell apoptosis, reducing inflammatory activity, and repressing cytoskeleton damage accompanied with alternations of podocyte specific proteins. Furthermore, functional assays substantiated that Nox4 was a direct target and negatively regulated by miR-423-5p. Additionally, restoration of Nox4 impeded the protective effect of miR-423-5p on podocyte injury via activation of p38 MAPK pathway. Therefore, this study manifested that miR-423-5p overexpression protected HG-induced podocyte damage by inhibiting ROS generation via targeting Nox4, providing a potential therapeutic strategy against DN.

1. Introduction

Diabetes nephropathy (DN) has become the major cause of severe end-stage renal disease and leads to the increasing morbidity and mortality worldwide [1]. The pathogenesis of DN is characterized by glomerular dysfunction, glomerulosclerosis, and decline in filtration barrier, of which podocyte injury has been considered to play critical role for the development of early DN [2]. Podocytes are highly differentiated epithelium with poor proliferative ability, which are mainly locate at the outer surface of the glomerular basement membrane (GBM) to constitute the glomerular filtration barrier [3]. High glucose (HG) has been demonstrated to contribute to podocyte injury and results in persistent proteinuria and declined glomerular filtration rate (GFR) in DN patients [4]. Therefore, it is urgently needed to identify novel and effective podocyte-based therapeutic strategies against DN.

microRNAs (miRNAs) as a class of single-stranded, short (21–25 nucleotides), and non-coding RNAs, could negatively regulate gene expressions at post-transcriptional level by complementarily binding to the 3′-untranslated regions (UTR) of target messenger RNAs (mRNAs) [5]. Recently, various miRNAs has been reported to be involved in the pathogenesis of various kidney diseases including DN [6]. miR-423-5p has been widely investigated as a novel biomarker in heart failure and human malignancies [7]. In kidney diseases, upregulation of miR-423-5p has been found in rat kidneys with ischemia/reperfusion injury and facilitates hypoxia-induced apoptosis in renal proximal tubular epithelial cells via endoplasmic reticulum stress [8]. In addition, highly expressed miR-423-5p is documented to be implicated in the pathogenesis of lupus nephritis [9]. However, the function of miR-423-5p in the pathogenesis of podocyte injury need to be further elucidated.

Accumulating evidence has demonstrated that excessive reactive oxygen species (ROS) production is responsible for the pathogenesis of various chronic kidney diseases, including DN [10]. Nicotinamide adenine dinucleotide phosphate oxidase 4 (Nox4) belongs to the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) family and is characterized by the constitutive release of hydrogen peroxide, which has been implicated as the major enzymatic source of ROS in diverse renal cells [11]. For example, podocyte-specific Nox4 deletion attenuated albuminuria

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https://doi.org/10.1016/j.bbrc.2018.09.067
0006-291X/

in association with reduction in extracellular matrix accumulation and ROS production in diabetic mice [12]. Furthermore, Nox1/4 inhibitor treatment has been shown to attenuate albuminuria and improve renal function in diabetic mice [13]. Therefore, these findings remain us to suppose that therapeutic targeting Nox4 might be considered as potential treatment strategy for diabetic complications.

Here, we demonstrated the protective role of miR-423-5p in HG-induced podocyte injury. Furthermore, the effect of and the potential mechanism involved in miR-423-5p/Nox4 pathway on regulating podocyte injury were explored in this study.

2. Material and method

2.1. Human kidney tissues collection

Renal biopsies from 15 adult cases of diagnosed with DN and 10 healthy controls were obtained from the Department of Nephrology in Xi'an Xidian Group Hospital. The written informed consent was received from all the patients. This study was approved by the Ethics Committee of Xi'an Xidian Group Hospital.

2.2. Immunohistochemistry and immunofluorescence staining

The renal tissues were formalin-fixed, paraffin-embedded and cut into 4 μm sections and then were deparaffinized, rehydrated, antigen recovery, and blocked. Subsequently, the slides were incubated with mouse anti-Nox4 and rabbit anti-synaptopodin (Abcam) overnight at 4 °C. For immunohistochemistry, the sections were incubated using DAB as substrate and counterstained with hematoxylin, and visualized by an invert microscopy (Olympus, Japan). For immunofluorescence staining, the slides were incubated with corresponding Alexa Fluor 488-conjugated donkey anti-rabbit and Alexa Fluor 594-conjugated donkey anti-mouse secondary antibodies (Invitrogen) in dark for 2 h at room temperature. Immunofluorescence images were taken under a confocal laser scanning microscope (Olympus, Japan).

2.3. Cell culture and treatment

The conditionally immortalized mouse podocyte cell line (MPC5) was obtained from the Cell culture Center of Peking Union Medical College (PUMC, Beijing, China). To stimulate podocyte proliferation, MPC5 cells were cultured in RPMI 1640 medium (Hyclone, Utah, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, MD, USA) containing 1% penicillin/streptomycin (Sigma-Aldrich, MO, USA) and 10 U/ml of interferon-γ (IFN-γ, Invitrogen, CA, USA) at the 33 °C in a humidified atmosphere of 5% CO_2. Subsequently, the podocytes were cultured in RPMI1640 complete medium without IFN-γ at 37 °C for 2 weeks to induce differentiation. After that, podocytes were treated with normal glucose (NG, 5 mM D-glucose) and high-glucose (HG, 30 mM D-glucose) for 12, 24, 48, and 72 h.

2.4. Oligonucleotide transfection

The miR-423-5p mimic (mimic) and the miR-scramble (Scr) were synthesized by GenePharma (Shanghai, China). To upregulation of Nox4 expression, the pcDNA3.1-Nox4 overexpressing recombinant plasmid was also constructed by GenePharma Co. And then, the oligonucleotides and plasmids were transfected into podocytes by using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions, followed by exposure to HG for 48 h, the transfection efficiency was measured by qRT-PCR or western blot. For inhibition of p38 MAPK, cells were pre-treated with p38 MAPK inhibitor SB203580 (10 μM, Sigma-Aldrich) for 2 h.

2.5. Quantitative RT-PCR

Total RNA was extracted by using TRIzol reagent (Invitrogen) following the manufacturer's instruction. To evaluate the expression of miR-423-5p, total RNA was reversely transcribed into cDNA by using One Step PrimeScript miRNA cNDA Synthesis Kit (Takara, Shanghai, China). For Nox4 expression, cDNAs was synthesized by using PrimeScript RT reagent Kit (Takara). And then, qRT-PCR reaction was performed on an ABI 7500 system (Applied Biosystems, CA, USA) using SYBR™ Green PCR Master Mix (Thermo Fisher Scientific, CA, USA) according to the manufacturer's protocol. β-actin and U6 were used as internal controls for normalization. The relative expression of miRNA or mRNA was calculated by using the $2^{-\triangle\triangle Ct}$ method.

2.6. Western blot analysis

Podocytes were lysed with RIPA lysis buffer (Beyotime, Beijing, China) and protein concentration was measured by the Bradford Assay Kit (Beyotime). An equal amount of proteins were separated in 12% SDS-PAGE gels, and then transferred onto PVDF membranes (Millipore, Billerica, CA, USA) by electroblotting. After blocking with 5% non-fat dry milk, the membranes were incubated with the primary antibodies (Abcam, Cambridge, USA) overnight at 4 °C. After rinsing, the protein bands were detected with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Abcam) and visualized using enhanced chemiluminescent reagents (Promega, Madison, WI, USA). Signals were quantified by Image J software (Bio-Rad, Hercules, CA, USA). β-actin served as internal control.

2.7. Cell viability assay

Cell viability was determined by CCK-8 method (Beyotime) according to the manufacturer's instruction. briefly, after transfection, MPC cells were seeded in a 96-well plate at the density of 1×10^4 per well and transfected with miR-423-5p mimic or NC for 48 h, and then 10 μl CCK-8 solution was added into each well and incubated for 1 h. The optical density (OD) values were measured at 490 nm using a microplate reader (Bio-Rad).

2.8. Apoptosis analysis

Cell apoptosis was detected by using AnnexinV-FITC Apoptosis Detection Kit (BD Biosciences, Franklin, NJ, USA) according to the manufacturer's protocols. Briefly, 1×10^5 podocytes were harvested, washed, centrifuged, and then resuspended in 500 μl 1 × binding buffer. 5 μl Annexin V-FITC and 5 μl propidium iodide (PI) regents were added and incubated for 15 min in the dark at room temperature. After that, cell apoptosis rate was analyzed by using a FASCalibur flow cytometer (BD Biosciences).

2.9. Measurement of intracellular ROS

Intracellular ROS production was determined by the 2′,7′-Dichlorodihydrofluorescin diacetate (DCFH-DA, Sigma-Aldrich). Podocytes with different treatment were incubated with 5 μM DCFH-DA for 30 min at 37 °C. After collecting and rinsing, the fluorescence intensity was detected at an emission wavelength of 530 nm and excitation wavelength of 485 nm by using FASCalibur (BD Biosciences).

Please cite this article in press as: Y. Xu, et al., miR-423-5p suppresses high-glucose-induced podocyte injury by targeting Nox4, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.09.067