Biochemical and Biophysical Research Communications xxx (2015) 1–6

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Silencing of angiotensin II type-1 receptor inhibits high glucose-induced epithelial–mesenchymal transition in human renal proximal tubular epithelial cells via inactivation of mTOR/p70S6K signaling pathway

Quanyou Gong*, Fangli Hou

Department of General Internal Medicine, Zhumadian City Center Hospital, Zhumadian 463000, Henan, China

ARTICLE INFO

Article history:
Received 12 November 2015
Accepted 20 November 2015
Available online xxx

Keywords:
Diabetic nephropathy
Tubulointerstitial fibrosis
Angiotensin II type-1 receptor
Epithelial–mesenchymal transition
mTOR/p70S6K

ABSTRACT

The epithelial–mesenchymal transition (EMT) plays a significant role in renal tubulointerstitial fibrosis (TIF), which is one of hallmark pathological feature of diabetic nephropathy (DN). Angiotensin II via its type-1 receptor AT1R is involved in the development of TIF. The purpose of our study was aimed to investigate the effect of silencing of AT1R on EMT and elucidate the possible mechanism underling these effects. EMT was induced by high glucose (HG) in human proximal tubular epithelial cell line HK-2 cells. The mRNA levels of AT1R were determined. The expression of AT1R was silenced by small interfering RNA (siRNA) technology and confirmed by quantitative real time PCR (qRT-PCR). After transfection with siAT1R, cell viability and expression levels of epithelial cell marker (epithelial (E)-cadherin), mesenchymal cell marker (alpha-smooth muscle actin (α-SMA)), four transcriptional factors (snail, slug, twist, and ZEB-1) were determined, as well as the roles of mechanistic target of rapamycin (mTOR)/p70S6K signaling pathway. The levels of AT1R were significantly higher after exposure to HG ($P < 0.05$). Transfection with siAT1R had no effect on cell viability, but reversed HG-induced EMT by up-regulation of E-cadherin expression and decrease of α-SMA, snail, and twist levels. MTOR/p70S6K signaling pathway was highly activated in HK-2 cells cultured under HG, but was inhibited by transfection with siAT1R. Our results suggest that silencing of AT1R inhibits EMT induced by HG in HK-2 cells via inactivation of mTOR/p70S6K signaling pathway. Silencing of AT1R might be a new strategy to treat DN.

1. Introduction

Diabetic nephropathy (DN) is one of the most serious complications in both type 1 and type 2 diabetes patients and the main cause of end-stage renal disease (ESRD) worldwide [1], which is recognized as a global public health problem in modern society. In spite of considerable attention has been devoted to prevent the progression and development toward chronic renal failure, the pathogenesis of DN is still unclear.

Morphologically, tubulointerstitial fibrosis (TIF) have been well demonstrated in the early stage of DN. Recently, a great number of studies have suggested that epithelial–mesenchymal transition (EMT) plays an important role in TIF [2–6]. The loss of epithelial specific markers, such as epithelial (E)-cadherin, and a gain of mesenchymal, such as marker alpha-smooth muscle actin (α-SMA) have been reported to be involved in EMT [7]. In consideration of the crucial role of EMT in initiating and promoting TIF in DN, a deeper understanding of the underlying mechanisms of EMT is critical to demonstrate the pathogenesis of DN and to provide a novel basis for the development of rational intervention strategies.

The renin angiotensin system (RAS) is also known to be associated with the genesis and progression of fibrotic diseases including a wide array of kidney diseases [8]. RAS exerts its functions via its main effector peptide angiotensin-II (Ang- II) and its transducer receptor AT1R [9]. In addition, administration of AT1R blockers (ARB) or AT1R antagonists has been shown to decrease fibrosis and has achieved considerable success in managing chronic kidney disease (CKD) [10–12]. Moreover, abnormal activation of mammalian target of rapamycin (mTOR)/S6 kinase 1 (S6K1)

* Corresponding author. Department of General Internal Medicine, Zhumadian City Center Hospital, No.747, Zhonghua Road, Yicheng District, Zhumadian 463000, Henan, China.
E-mail address: gongquanyouxh0004@126.com (Q. Gong).

http://dx.doi.org/10.1016/j.bbrc.2015.11.092
0006-291X/

signaling has been implicated in the pathogenesis of TIF [13]. An important role of AT1R signaling in promotion of fibrosis in TIF through activation of the mTOR/S6K1 signaling pathway has been supported [14]. However, rare studies have been determined the functions of AT1R specifically on EMT and whether mTOR signaling pathway is responsible for these effects.

Therefore, the purpose of our study was to explore the effects of AT1R on EMT in renal tubular epithelial cells and TIF in DN and try to clarify the possible mechanism underlying these effects. Our study might provide new sights into the development of strategies to treat DN.

2. Material and methods

2.1. Cell culture and treatment

The human proximal tubular epithelial cell line HK-2 was obtained from American Type Culture Collection (ATCC, Rockville, MD). The cells were grown in Dulbecco's modified Eagle's medium (DMEM)/F12 medium (Gibco; Life Technologies, Carlsbad, CA) containing 5.56 mmol/L D-glucose (normal glucose, NG), supplemented with 10% fetal bovine serum (FBS, Life Technologies), 100 U/ml penicillin (Life Technologies), 100 μg/ml streptomycin (Life Technologies) at 37 °C in a humidified atmosphere of 5% CO_2. All experiments were performed with confluent cultures. The confluent cells were cultured in serum-free DMEM medium for 24 h before the experiments. The cells were exposed to high glucose (HG) medium (60 mmol/L D-glucose) for 72 h to induce EMT. The cells subjected to NG were used as a control.

2.2. Transfection of siRNA

To knockdown the gene expression of AT1R, the siRNA expressing plasmids specifically targeting AT1R and control siRNA (no silencing) were designed, synthesized, and supplied by GenePharma Co (Shanghai, China). The confluent cells were cultured in serum-free DMEM medium for 24 h before the transfection. Transfection was conducted in 24-well plates with 40 nM siRNAs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. After 48 h incubation with siAT1R, the cells were harvested for further analyses. AT1R knockdown was confirmed by quantitative real time PCR (qRT-PCR).

2.3. RNA isolation and qRT-PCR

Total mRNA was isolated from HK-2 cells using Trizol reagent (Life Technologies) according to the manufacturer's instructions. Complementary DNA (cDNA) was produced using 1 μg of total RNA and reverse transcribed using the SuperScript II reverse transcriptase kit (Invitrogen, Paisley, UK). The expression levels of AT1R, E-cadherin,α-SMA, snail, slug, twist, and ZEB-1 were measured by SYBR green based qRT-PCR (SYBR Green PCR Master Mix, Applied Biosystems, Carlsbad, CA), and relative gene expression was determined by normalizing to GAPDH using the $2^{-\Delta\Delta CT}$ method.

2.4. Cell proliferation

The survival of HK-2 cells was assessed using 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide (MTT) colorimetric assay. HK-2 cells (5×10^4 cells/ml) transfected with or without siAT1R were harvested, washed with phosphate buffered saline (PBS), seeded in 96-well plate, and incubated with fresh medium for 24 h. At different time points (24 h, 48 h, 72 h, and 96 h incubation), MTT (10 μL) was added to each well at a final concentration of 0.5 mg/mL. The cells were incubated for another 4 h at 37 °C. The absorbance at 570 nm was measured using a Synergy 4 Multi-Mode Microplate Reader (BioTek Instruments, Bad Friedrichshall, Germany).

2.5. Western blot analysis

HK-2 cells were harvested and washed twice with PBS. Cell lysates were centrifuged at 4 °C for 10 min at 12,000 g, and the protein concentration was quantified using the Bio-Rad protein assay (Bio-Rad, Helmel Hempstead, UK). The protein samples (50 μg) were separated by a 10–12% sodium dodecyl sulfate (SDS)-polyacrylamide gel (PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were then blocked with 5% nonfat dry milk for 2 h at room temperature and incubated at 4 °C overnight with primary antibodies against the following target proteins: snail, slug, twist, ZEB-1, the phosphorylation of mTOR at Ser-2448 (p-mTOR$^{Ser\text{-}2448}$), p-mTOR, the phosphorylation of p70S6K at Thr-389 (p-p70S6K$^{Thr\text{-}389}$), p70S6K or β-actin. All the antibodies were purchased from Cell Signaling Technology (Beverly, MA). The membranes were then washed twice with PBS-Tween and incubated with appropriate peroxidase-conjugated secondary antibodies (Cell Signaling Technology) for 2 h at room temperature. Enhanced chemiluminescence system (Amersham Pharmacia Biotech) was used to detect the specific bands. Band densities of resulting autoradiograms were quantified by scanning densitometry (GS-800 Imaging Calibrated Densitometer, Bio-Rad). Relative protein levels were standardized to β-actin levels.

2.6. Statistical analysis

All data were expressed as the means ± standard deviation (SD). The comparisons between groups were analyzed using one-way analysis of variance (ANOVA). Statistical analyses were performed using Statistical Package for the Social Science (SPSS) 19.0 statistical software. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Higher expression of AT1R by HG-induced EMT in HK-2 cells

To investigate the expression of AT1R by HG-induced EMT in HK-2 cells, we exposed HK-2 cells to NG or HG for 72 h. The mRNA expression levels of AT1R in HK-2cells were detected. The results showed that the mRNA expression levels of AT1R were significantly higher after exposure to HG than those exposure to NG ($P < 0.05$), demonstrating that AT1R was involved in the EMT induced by HG in HK-2 cells (Fig. 1).

3.2. Expression of AT1R in HK-2 cells after AT1R-specific siRNA transfection

To determine the roles of AT1R in EMT induced by HG in HK-2 cells, AT1R-specific siRNA was performed to selectively silence the expression of AT1R. QRT-PCR was carried out to determine the knockdown efficiency at 48 h after transfection. As shown in Fig. 2, the expression of AT1R was significantly decreased by transfection with the AT1R-specific siRNA in HK-2 cells compared with the negative control group ($P < 0.05$).

3.3. Effects of AT1R-specific siRNA transfection on cell viability

The MTT assay was performed to quantify the effects of AT1R-specific siRNA transfection on HK-2 cell viability. The cell viability was assessed at different time points after transfection with siAT1R

Please cite this article in press as: Q. Gong, F. Hou, Silencing of angiotensin II type-1 receptor inhibits high glucose-induced epithelial–mesenchymal transition in human renal proximal tubular epithelial cells via inactivation of mTOR/p70S6K signaling pathway, Biochemical and Biophysical Research Communications (2015), http://dx.doi.org/10.1016/j.bbrc.2015.11.092