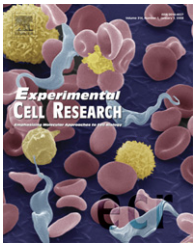
EXPERIMENTAL CELL RESEARCH 314 (2008) 3654–3662

available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/yexcr



Research Article

Aldosterone induces myofibroblastic transdifferentiation and collagen gene expression through the Rho-kinase dependent signaling pathway in rat mesangial cells

Suwarni Diah[a], *Guo-Xing Zhang*[a], *Yukiko Nagai*[b], *Wei Zhang*[c], *Liu Gang*[a], *Shoji Kimura*[a], *Mas RW Abdul Hamid*[d], *Takashi Tamiya*[c], *Akira Nishiyama*[a], *Hirofumi Hitomi*[a,*]

[a]*Department of Pharmacology Faculty of Medicine, Kagawa University, 761-0793, Japan*
[b]*Life Sciences Research Center, Faculty of Medicine, Kagawa University, 761-0793, Japan*
[c]*Department of Neurological Surgery, Faculty of Medicine, Kagawa University, 761-0793, Japan*
[d]*Institute of Medicine, Universiti Brunei Darussalam, Jalan Tungku Link, Gadong BE1410, Brunei Darussalam*

ARTICLE INFORMATION

Article Chronology:
Received 16 April 2008
Revised version received
16 August 2008
Accepted 13 September 2008
Available online 7 October 2008

Keywords:
Aldosterone
Rho-kinase
Eplerenone
Y27632
Myofibroblastic transdifferentiation
Cell hypertrophy
Collagen
α-smooth muscle actin (α-SMA)
Actin polymerization
Rat mesangial cel (RMCs)

ABSTRACT

There is accumulating evidence indicating the role of aldosterone in the pathogenesis of hypertension and renal injury. In this study, we investigated the role of the Rho-kinase dependent signaling pathway in aldosterone-induced myofibroblastic transdifferentiation and collagen gene expression in rat mesangial cells (RMCs). Stimulation with aldosterone (1 nmol/L) significantly increased phosphorylation of myosin phosphatase target subunit-1 (MYPT-1), a marker of Rho-kinase activity, with a peak at 20 min in RMCs. Pre-incubation with a selective mineralocorticoid receptor antagonist, eplerenone (10 μmol/L), or a specific Rho-kinase inhibitor, Y27632 (10 μmol/L), attenuated the aldosterone-induced increase in MYPT-1 phosphorylation. Aldosterone also induced hypertrophy in RMCs, accompanied by an increase in actin polymerization and expression of α-smooth muscle actin (α-SMA), a myofibroblastic transdifferentiation marker. Collagen type I, III and IV mRNA levels were also increased with aldosterone stimulation. Pre-treatment with eplerenone or Y27632 prevented the aldosterone-induced cell hypertrophy, actin polymerization, the increase in α-SMA expression and the increases of collagen type I, III, IV mRNA levels in RMCs. These results suggest that aldosterone-induced mesangial cell hypertrophy is associated with cell transformation, leading to an increase in collagen gene expression via the Rho-kinase dependent signaling pathway.

Introduction

Evidence has accumulated supporting the potential role of aldosterone in the pathogenesis of cardiovascular disease and renal injury [1–3]. Treatment with mineralocorticoid receptor (MR) antagonists ameliorates renal injury in stroke-prone spontaneously hypertensive rats [4] and Dahl salt-sensitive hypertensive rats [5–6]. Further studies show that treatment with aldosterone

* *Corresponding author.* Department of Pharmacology, Faculty of Medicine, Kagawa University, 1750-1 Ikenobe, Miki-cho, Kita-gun, Kagawa, 761-0793, Japan. Fax: +81 87 891 2126.
E-mail address: hitomi@kms.ac.jp (H. Hitomi).

0014-4827/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexcr.2008.09.018

induces severe glomerular injury characterized by mesangial matrix expansion in rats [7–9]. Clinical studies have also shown that treatment with MR antagonists reduces albuminuria in patients with diabetic nephropathy [10–11] or chronic kidney disease [12]. However, the precise mechanisms responsible for aldosterone-induced renal injury remain unexplored.

Rho-kinase is an important molecule that mediates various cellular functions such as contraction, adhesion, proliferation, motility or migration, cellular morphology, growth control and cytokinesis [13–14]. Several studies have demonstrated the potential involvement of Rho-kinase in the pathogenesis of renal injury [5,15]. The selective Rho-kinase inhibitor, Y27632, has been shown to attenuate tubulointerstitial fibrosis in mouse kidneys with unilateral urethral obstruction [16]. Fasudil, another Rho-kinase inhibitor, ameliorates glomerulosclerosis in Dahl salt-sensitive rats [17]. We recently demonstrated that treatment with fasudil markedly ameliorated aldosterone-induced glomerular mesangial injury, independent of blood pressure changes [18]. Taken together, these findings suggest that aldosterone-induced glomerular mesangial injury is involved in Rho-kinase dependent signaling pathways. However, there is no convincing evidence that supports the role of Rho-kinase in aldosterone-induced mesangial cell hypertrophy.

Recently, it has been proposed that renal fibrosis is associated with phenotypic transitions: myofibroblast transdifferentiation (MFT; for non-epithelial cells) and epithelial–mesenchymal transition (EMT; for epithelial cells) [19]. Profibrotic mediators, such as transforming growth factor-β, connective tissue growth factor, angiotensin II, endothelin-1, and fibroblast growth factor have been shown to induce MFT or EMT [20–22]. Aldosterone-mediated induction of EMT in renal epithelial (proximal tubular) cells has been recently reported [23]. However, whether aldosterone induces mesangial cell hypertrophy through MFT and its underlying mechanisms remained unclear.

In this study, we investigated the potential roles of Rho-kinase in aldosterone-induced mesangial cell hypertophy. In particular, we investigated whether Rho-kinase is involved in aldosterone-induced MFT and collagen expression in rat mesangial cells (RMCs).

Materials and methods

Cell culture

RMCs were obtained from Sprague–Dawley rats and maintained as previously reported [24–25]. Control solutions contained the appropriate amount of vehicle: ethanol for aldosterone (Wako, Co., Osaka, Japan), dimethyl sulfoxide for eplerenone (Pfizer, Inc. New York, NY), dimethyl sulfoxide for latrunculin B (Calbiochem, La Jolla, CA), and distilled water for Y27632 (Calbiochem, La Jolla, CA). In this experiment, cells were used between passage 5 and 12. After stimulation with the above-mentioned compounds, protein or mRNA was extracted as described previously [24]. Protein concentrations were determined using protein assay kits (Bio-Rad, Hercules, CA).

The effect of aldosterone on Rho kinase activity

To evaluate Rho-kinase activity in RMCs after aldosterone stimulation, two different methods were used: ELISA and Western blotting with specific antibodies against phospho-MYPT-1. Rho-kinase activity can be assayed by measuring the phosphorylation levels of MYPT-1, also known as myosin binding subunit (MBS) [26–30]. In brief, levels of phosphorylated MYPT-1 were determined using an ELISA kit with a monoclonal antibody (CycLex Co., Nagano, Japan) specific for the phosphorylated form of MYPT-1 (phosphorylated at Thr-696) according to the manufacturer's instructions. Immunoblotting was performed with a primary antibody against phospho-MYPT-1 Thr-696 (1:500 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C overnight. After washing, an anti-rabbit secondary antibody (1:20,000 in 3% skimmed milk, Cell Signaling Technology Inc., Danvers, MA) was added for 1 h at room temperature. Total MYPT-1 and β-actin protein expression were also assessed with a primary antibody against MYPT-1 (Santa Cruz Biotechnology) or β-actin (Sigma Chemical Co.), respectively. Western blotting data were quantified by densitometric analysis (NIH Image software).

Assessment of mesangial cell hypertrophy

Cell hypertrophy was assessed by examining RMCs surface area and protein/cell number ratio. Using a light microscope, more than 7 fields of each sample were observed and individual cell area was examined by NIH image software. Cells were divided into four groups according to their surface area (<100, 100–200, 200–300 and >300 μm^2). Data were expressed as percentage of cell number in each group after treatment. To examine the protein/cell number ratio, protein concentration was measured using the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA) and cell number was counted for 6 different fields for each group.

Assessment of α-smooth muscle actin (α-SMA) expression

α-SMA expression was determined using immunofluorescence and Western blotting analysis. For immunofluorescence, RMCs were grown on 2-well glass chamber slides (BD Bioscience, NJ, USA). Following stimulation, cells were fixed using a cold methanol/acetone solution. The slides were then blocked using 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 1 h at 37 °C in a humidified chamber. The slides were incubated with mouse monoclonal antibody against α-SMA (1:200 dilution, Sigma Chemical Co.) for 2 h at 37 °C in the humidified chamber. After washing with PBS, cells were incubated with anti-mouse IgG FITC-conjugate (1:500 dilution, Sigma Chemical Co.) for 1 h at 37 °C in the humidified chamber. Cells were washed briefly prior to cell fluorescence intensity assessment using a Radiance 2100 confocal microscope (Bio-Rad, Hercules, CA). For α-SMA western blotting analysis, an α-SMA mouse monoclonal primary antibody (Sigma Chemical Co.) was used as the primary antibody.

F-actin/G-actin assay

Actin polymerization was analyzed by determining the ratio of filamentous actin (F-actin) content vs. free globular-actin (G-actin) content, using the F-actin/G-actin *in vivo* Assay kit (Cytoskeleton Inc., Denver, USA) according to manufacturer's instructions [31].

Real time RT-PCR

The mRNA levels of MR, collagen types I, III, and IV and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were analyzed using real