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Schizandrin prevents damage of murine mesangial cells via blocking NADPH oxidase-induced ROS signaling in high glucose

Seung-Il Jeong, Sang-Jun Kim, Tae-Ho Kwon, Kang-Yeol Yu, Seon-Young Kim *

Jeonju Biomaterials Institute, Jeonju, Chonbuk, Republic of Korea

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ABSTRACT

High glucose (HG) is the underlying factor contributing to long term complication of diabetes mellitus. Reactive oxygen species (ROS) have been postulated as a unifying mechanism for HG-induced complications. NADPH oxidase, producing superoxide anion, is the main source of ROS in diabetic nephropathy. In this study we report the inhibitory effect of schizandrin (Sch), an active ingredient of *Fructus schisandrae*, on HG-induced murine mesangial cells (MMCs) damage. Sch treatment significantly attenuated HG-induced proliferation and protein synthesis of MMCs in a dose dependent manner. The intracellular reactive oxygen species (ROS) level was also remarkably reduced by Sch as well as the enhanced NADPH oxidase activity, superoxide anion levels, NOX4 and p22phox protein expression, and phosphorylation of p47phox and p67phox. The phosphorylation level of mitogen activated kinase (MAPK) protein, phospho-Erk1/2 and -p38, and Akt was also significantly inhibited by Sch under HG condition. By using specific inhibitors, we found that Sch inhibits HG-induced mesangial cell proliferation and ECM overexpression via NADPH oxidase/PI3K–Akt–MAPK-dependent pathway in MMCs. Taken together; our demonstration of the ability of Sch to inhibit high glucose induced damage of MMCs has clinical implications in treatment of diabetic nephropathy.

1. Introduction

Diabetic nephropathy (DN) is a leading cause of end-stage renal disease, which is characterized by excessive deposition of extracellular matrix (ECM) in kidney, causing glomerular mesangial expansion and fibrosis (Gilbert and Cooper., 1999). Though the precise mechanisms of DN have not been elucidated, several *in vivo* studies have recently unraveled that mesangial cell proliferation and mesangial expansion is one of the early key pathologic features of diabetic nephropathy (Gorin et al., 2005; Kim et al., 2009). It seems that mesangial hypercellularity precedes an increase in the extracellular matrix proteins and glomerular sclerosis, hallmarks of diabetic nephropathy (Danesh et al., 2002. HG induces reactive oxygen species (ROS), which play a key role in the glomerular mesangial cells hyperplasia and amplify glucose signaling (Ayo et al., 1990; Ha and Lee, 2000).

Although multiple enzymes contribute to oxidative stress in different tissues or cells, a number of reports have indicated that NADPH oxidase derived superoxide is central to hyperglycemia induced oxidative stress in DN (Paravicini and Touyz, 2008; Griendling et al., 2000). Phagocytic NADPH oxidase consists of a catalytic core of two membrane-associated subunits, gp91phox and p22phox. NADPH oxidase in the resting state becomes activated to produce superoxide upon interaction with cytoplasmic components, p47phox and p67phox, and Rac, a small GTPase. Non-phagocytic cells use a similar machinery to generate superoxide (Bokoch and Knaus, 2003). Interestingly, mesangial cells express all components required for functional NADPH oxidase systems, except from the catalytic subunit gp91phox (Jones et al., 1995). Therefore, the existence of one or more alternative catalytic subunits in these cells was suspected to replace gp91phox. In fact, Geiszt et al. (2000) reported a kidney NADPH oxidase isoform that initially termed renal NOX (Renox). Meanwhile, some other gp91phox homologues from different tissues and cells were identified, and the nomenclature was accommodated. MOX1, gp91phox, and Renox are now termed NOX1, NOX2, and NOX4, respectively (Lambeth, 2004). Increases in NOX4 levels were detected in the kidney of diabetic rats 4–8 weeks after the onset of diabetes with similar increases detected in mesangial cells exposed to high glucose (Yi et al., 2006; Gorin et al., 2005).

The serine/threonine kinase Akt regulates a number of cellular functions, including glucose metabolism, glycogen synthesis,

Abbreviations: DN, diabetic nephropathy; DCF, 2′,7′-dichlorofluorescein; DMEM, Dulbecco's modified Eagle's medium; DPI, diphenylene iodonium; ECM, extracellular matrix; Erk1/2, extracellular signal-regulated kinase 1/2; HG, high glucose; HM, high mannitol; MAPK, mitogen activated kinase; MMCs, murine mesangial cells, MES-13 cells; MTS, 3,4-(5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; NADPH, nicotinamide adenine dinucleotide phosphate; PBS, phosphate-buffered saline; PI3K, phosphoinositide 3-kinase; ROS, reactive oxygen species.

* Corresponding author. Tel.: +82 63 711 1053; fax: +82 63 711 1051.
E-mail address: Seon02@jbmi.re.kr (S.-Y. Kim).

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protein synthesis, cell proliferation, cell hypertrophy, and cell death, which is one of the downstream effectors of phosphoinositide 3-kinase (PI3K) (Shiojima and Walsh, 2002; Gorin et al., 2005). Our recent report demonstrated that Akt is an important mediator of mesangial cell proliferation and ECM protein accumulation (Kim et al., 2008). We also reported that Akt is activated in renal damage in streptozotocin-induced diabetic mice (Kim et al., 2009).

The mitogen-activated kinases (MAPKs) are also unregulated in renal cells by hyperglycemia (Fujita et al., 2004; Kim et al., 2009). Studies have shown that the activation of p38 and extracellular signaling kinase (Erk) 1/2 are participating in renal tubular cells and mesangial cells proliferation in high glucose (Fujita et al., 2004; Jia et al., 2009).

Schizandrin (Sch), the major lignan isolated from the *Schisandra chinensis*, possesses many biological properties including hepatoprotective (Ip et al., 1995), anti-inflammatory (Guo et al., 2008), antitumor (Huang et al., 2008), and anti-asthmatic activities (Lee et al., 2010). In the kidney, Sch have shown the protective effect on gentamicin-induced nephrotoxicity against oxidative stress (Chiu et al., 2008). However, signaling mechanisms for therapeutic effects of Sch on the mesangial cells damage induced by HG have not been reported. In this study, we investigated the effect of Sch on HG-mediated mesangial cell proliferation and ECM protein overproduction through a mechanism associated with the inhibition of NADPH oxidase-mediated ROS production, PI3K/Akt, and Erk 1/2 and p38 MAP kinase activation in hyperglycemic condition.

2. Materials and methods

2.1. Chemicals and antibodies

Phoshate-buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and antibiotics (amphotericin B, penicillin, and streptomycin) were purchased from Invitrogen (Carlsbad, CA, USA). Dimethyl sulfoxide (DMSO), 2′,7′-dichlorofluorescein diacetate (DCF-DA), diphenylene iodonium (DPI), LY294002, PD98059, SB203580, and other chemicals were obtained from Sigma (St. Louis, Mo, USA). Schizandrin (Sch) was isolated from the petroleum extract of *Fructus schisandrae* and identified by comparison of spectroscopic data with the previous report (Ikeya et al., 1979). The purity of Sch was >95%, as determined by HPLC analysis (Ip et al., 1995). Antibodies were obtained as following sources:anti-phospho-Akt (Ser473) monoclonal antibody (mAb), anti-Akt polyclonal antibody (pAb), anti-Erk1/2 (Thr202/Tyr204) pAb, and anti-Erk 1/2 rabbit pAb from Cell Signaling Technology (Beverly, MA); anti-phosphoserine mAb from Abcam (Cambridge, UK); anti-phospho-p38 pAb, anti-p38 mAb, anti-NOX4 pAb, anti-p47phox pAb, anti-p67phox pAb, anti-p22phox pAb, anti-collagen IV pAb, anti-TGF-β1 pAb, and anti-fibronectin pAb, and horseradish peroxidase (HRP)-conjugated anti-mouse IgG, anti-goat IgG, and anti-rabbit IgG were purchased from Santa Cruz Biotechnology (SantaCruz, CA).

2.2. Culture of MMCs

SV40-transformed MMCs (MES-13) were obtained from America Type Culture Collection (ATCC; Rockville, MD, USA) and maintained in DMEM containing 5% FBS, 0.25 μg/ml amphotericin B, 100 U/ml penicillin, and 100 U/ml streptomycin at 37 °C in 5% CO_2, 95% air. Cells were passaged three times per week.

2.3. Proliferation assay

Cells were seeded at a density of 1.5×10^4 cells/well in a 96-well plate. When the cells reached 60–70% confluence, the growth medium was aspirated and the wells were rinsed with pre-warmed PBS. Quiescent cells were exposed to a fresh medium with different concentrations of Sch (0.1–100 μM) or 0.1% DMSO (vehicle control) for 1 h. After incubation, 20 μl of a solution of CellTiter 96 Aqueous One Solution (Promega, Madison, WI, USA) containing MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] and an electron coupling reagent (phenazine ethosulfate) were added to each well. The plates were incubated for 3 h during which time the reagent was bio-reduced into a colored formazan product by the intracellular dehydrogenase enzymes of metabolically active cells. The absorbance (A) was measured at 490 nm (Perkin Elmer, Victor^2 1420 Multilabel counter).

2.4. Determination of DNA synthesis

A total of 1×10^4 MMCs/well were seeded onto 6-well plates and grown to semiconfluence in DMEM containing a normal glucose concentration and 5% FBS for 24 h. Cells were washed once with PBS before growth arresting in DMEM without FBS for 48 h. Quiescent MCs were stimulated with HG and pretreated with different concentrations of Sch (0.1–100 μM) for 48 h. DNA synthesis was quantified by 5-bromo-2′-deoxyuridine (BrdU) incorporation into proliferating cells over 2 h (Roche Diagnostics, Mannheim, Germany).

2.5. Total protein to cell count ratio

The ratio of total protein content to cell number is another well established measure of cellular hypertrophy (Wolf et al., 2001). To measure this ratio, MMCs were seeded into each well of a six-well plate and were synchronized into quiescence for 12 h in serum free medium containing a normal glucose concentration. MCs were stimulated with HG and pretreated with different concentrations of Sch (0.1–100 μM) for 48 h. After incubation, cells were trypsinized, scraped off the plate with a rubber policeman, and washed twice in PBS. A small aliquot of cells were used for cell counting of intact viable cells after trypan blue (Biochrom, Germany) staining using a haemocytometer to calculate the cell number/protein ratio. The remaining cells were lysed in RIPA buffer (10 mM Tris/HCl (pH 7.4), 150 mM NaCl, 10 mM EGTA, 10 mM EDTA, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 1% (v/v) Triton X-100, 20 μM leupeptin, 1 mM PMSF and 0.15 μM pepstatin), and total protein content was measured by a Bradford method. Total protein content was expressed as microgram protein per 10^4 cells. These experiments were independently carried out three times.

2.6. Measurement of ROS

Generation of intracellular ROS was measured with the fluoroprobe DCF-DA. Briefly, MMCs were incubated for 60 min at 37 °C with 10 μmol/l DCF-DA in absence or presence of Sch in a dose-dependently. Fluorescence intensity was measured by fluorescence microscopy (NIKON; excitation 488 nm, emission 513 nm). Average intensity for each experimental group of cells was determined using Scion Image Analysis software, and values were expressed as above control.

2.7. NADPH oxidase activity

NADPH oxidase activity was measured by the lucigenin-enhanced cehmiluminescence method as previously described (Gorin et al., 2005). MMCs were stimulated with glucose for 1 h after treat Sch for 30 min. The cells were washed and homogenized in lysis buffer containing 20 mM KH_2PO_4 (pH 7.0), 1 mM EGTA, 1 mM PMSF, 10 μg/ml aprotinin, and 0.5 μg/ml leupeptin. A total of 100 μl of homogenate was immediately added to 900 μl of 50 mM phosphate buffer (pH 7.0) containing 1 mM EGTA, 150 mM sucrose, 5 μM lucigenin as an electron acceptor, and 100 μM NADPH as an electron donor. Photon emission expressed as relative light units was measured every 30 s for 5 min in luminometer. There was no measurable activity in the absence NADPH. A buffer blank (<5% of cell signal) was subtracted from each reading before calculation of the data. Superoxide production was expressed as the rate of relative chemiluminescene (Perkin Elmer, Victor^2 1420 Multilabel counter) units. Protein content was measured using the Bio-Rad protein assay reagent. NADPH oxidase activity experiments were also repeated three times, each showing same trends. Data are illustrated in representative graphs.

2.8. Determination of superoxide anion levels

Superoxide anion production by mesangial cells was determined by measuring the superoxide dismutase-inhibitable reduction of cytochrome c with a spectrophotometer as previously described (Chen et al., 1997). Briefly, the mesangial cells were grown in 24-well tissue culture plates. The cells were treated with various inhibitors, containing cytochrome c (80 μM) with or without superoxide dismutase (100 μg/ml). The absorbance was then measured with a spectrophotometer at 550 nm. The relative amount of superoxide anions secreted by cells was calculated.

2.9. Immunoblotting

Protein extraction and immunoblotting of MMCs were performed as previously described (Kim et al., 2008). Proteins (20 μg per lane) were resolved on a 10% or 12% SDS–PAGE gel and transferred to polyvinylidene difluoride (PVDF, GE Healthcare, Little Chalfont, Buckinghamshire, UK) membranes. Blots were incubated with primary antibodies (1:2500 dilution of each antibody) overnight at 4 °C. The blots were rinsed four times with blocking buffer and incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000 dilutions of each antibody) for 1 h at room temperature. The binding of the antibodies was visualized using an enhanced chemiluminescence (ECL) system (Bio-Rad, Munich, Germany). Protein concentrations were determined using a Bio-Rad protein assay kit, and known