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High glucose induces renal mesangial cell proliferation and fibronectin expression through JNK/NF-κB/NADPH oxidase/ROS pathway, which is inhibited by resveratrol

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ABSTRACT

Renal hypertrophy and extracellular matrix accumulation are early features of diabetic nephropathy. Hyperglycemia-induced oxidative stress is implicated in the etiology of diabetic nephropathy. Resveratrol has potent antioxidative and protective effects on diabetic nephropathy. We aimed to examine whether high glucose (HG)-induced NADPH oxidase activation and reactive oxygen species (ROS) production contribute to glomerular mesangial cell proliferation and fibronectin expression and the effect of resveratrol on HG action in mesangial cells. By using rat mesangial cell line and primary mesangial cells, we found that NADPH oxidase inhibitor (apocynin) and ROS inhibitor (N-acetyl cysteine) both inhibited HG-induced mesangial cell proliferation and fibronectin expression. HG-induced elevation of NADPH oxidase activity and production of ROS in mesangial cells was inhibited by apocynin. These results suggest that HG induces mesangial cell proliferation and fibronectin expression through NADPH oxidase-mediated ROS production. Mechanistic studies revealed that HG upregulated NADPH oxidase subunits $p22^{phox}$ and $p47^{phox}$ expression through JNK/NF-κB pathway, which resulted in elevation of NADPH oxidase activity and consequent ROS production. Resveratrol prevented HG-induced mesangial cell proliferation and fibronectin expression through inhibiting HG-induced JNK and NF-κB activation, NADPH oxidase activity elevation and ROS production. These results demonstrate that HG enhances mesangial cell proliferation and fibronectin expression through JNK/NF-κB/NADPH oxidase/ROS pathway, which was inhibited by resveratrol. Our findings provide novel therapeutic targets for diabetic nephropathy.

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1. Introduction

Diabetic nephropathy is an important complication of type 1 and type 2 diabetes. It is characterized by an expansion of the glomerular mesangium, caused by mesangial cell proliferation and an excessive accumulation of extracellular matrix (ECM) proteins synthesized by mesangial cells. Clinical and animal studies show the increase in the markers of oxidative stress or in production of reactive oxygen species (ROS) in diabetic kidney (Ha et al., 2008). There is increasing evidence that overproduction of ROS is one of the major factors in the development of diabetic nephropathy (Ha et al., 2008; Forbes et al., 2008). ROS activates protein kinase C, mitogen-activated protein (MAP) kinase, and transcription factors (NF-κB, activated protein-1) which eventually result in altered expression of genes and ECM proteins leading to diabetic nephropathy (Kashihara et al., 2010). NADPH oxidase is an important source of ROS production. The phagocyte NADPH oxidase consists of the membrane-associated subunit $p22^{phox}$ and Nox2 (originally named $gp91^{phox}$), the cytosolic regulatory subunits $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and the GTPase Rac1 (Gill and Wilcox, 2006). There are six homologues of phagocytic Nox2 proteins expressed by distinct tissues. Human and rat glomerular mesangial cells express $p22^{phox}$, $p47^{phox}$, $p67^{phox}$ and Nox4 subunits of NADPH oxidase (Gill and Wilcox, 2006). The expression of subunits of NADPH oxidase increases in experimental models of diabetic nephropathy, and inhibition of NADPH oxidase with apocynin or diphenylene iodinium decreases renal ROS production and

Abbreviations: ECM, extracellular matrix; H_2DCF-DA, 2′ 7′-dichlorodihydrofluorescein diacetate; HG, high glucose; MAP, mitogen-activated protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, N-acetyl cysteine; NG, normal glucose; ROS, reactive oxygen species.

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ameliorates renal morphological changes and functional abnormalities (Asaba et al., 2005; Nam et al., 2009). Nox4-based NAD(P)H oxidase has been reported to be involved in kidney and glomerular hypertrophy and fibronectin accumulation in animal model of diabetic nephropathy (Gorin et al., 2005; Sedeek et al., 2010). These observations suggest that NADPH-derived ROS is one of the major mediators in the pathogenesis of diabetic nephropathy.

Hyperglycemia plays a central role in the development and progression of diabetes nephropathy (Kikkawa et al., 2003). High glucose promotes mesangial cell proliferation and fibronectin expression in vitro (Yano et al., 2009; Ayo et al., 1990). It induces ROS production in mesangial cells through activation of NADPH oxidase and mitochondrial metabolism (Lee et al., 2003). However, the mechanisms of NADPH oxidase activation/upregulation by high glucose are not fully understood. Whether NADPH oxidase-derived ROS contributes to high glucose-enhanced mesangial proliferation is unclear.

Resveratrol (3,5,4′-trihydroxystilbene) is a polyphenolic phytoalexin that occurs naturally in many plant species, including grapevines and berries, and exhibits pharmacologic health benefits including antioxidative (Leonard et al., 2003), anti-inflammatory (Zhao et al., 2011), anticancer (Aggarwal et al., 2004) and cardioprotective properties (Das and Das, 2007). Resveratrol has been reported to ameliorate hyperglycemia and hyperlipidemia in diabetic animal models (Su et al., 2006; Palsamy and Subramanian, 2011). Recent studies with rodent diatetic models showed that resveratrol inhibited oxidative stress in kidney, attenuated glomerular fibronectin/collagen IV expression and mesangial matrix expansion, and ameliorated renal function (Palsamy and Subramanian, 2011; Sharma et al., 2006; Chen et al., 2011; Kitada et al., 2011). However, it is unclear whether resveratrol could inhibit high-glucose induced mesangial cell proliferation and fibronectin expression through its anti-oxidative activity.

In the present study we investigated whether NADPH oxidase-derived ROS is involved in high glucose-induced mesangial cell proliferation and fibronectin production, and explored the mechanisms underlying NADPH oxidase activation by high glucose in mesangial cells. We also examined the effect of resveratrol on high glucose-induced mesangial cell proliferation and fibronectin expression, and explored the mechanisms involved.

2. Materials and methods

2.1. Reagents

Resveratrol, apocynin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and N-acetyl-L-cysteine were obtained from Sigma (St Louis, MO). 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) was from Invitrogen (Carlsbad, CA). Antibodies against $p22^{phox}$ and $p47^{phox}$ were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against fibronectin and β-actin were from ABCAM (ABCAM, Cambridge, UK). Antibodies against phosphorylated IκBα and total IκBα were from Cell Signaling Technology (New England Biolabs, Beverly, MA).

2.2. Mesangial cell culture

Rat renal mesangial cell line (CRL-2573, ATCC) was cultured in DMEM containing 5.6 mM (1000 mg/l) glucose, 10% FBS (v/v), 100 U/ml penicillin and 100 μg/ml streptomycin (Bio Basis Inc., Markham, ON, Canada). High glucose treatment was performed by culturing cells in DMEM containing 25 mM (4500 mg/l) glucose for the indicated times.

Primary rat mesangial cells were isolated from Sprague-Dawley rat kidney glomeruli with type IV collagenase (Luo et al., 2006). Cells were confirmed as mesangial cells by their typical morphology and positive immunostaining against smooth muscle α-actin (data not shown). Mesangial cells were grown in DMEM supplemented with 20% FBS and antibiotics. Passages 3–10 were used in the experiments. All experiments using animals were approved by the Animal Care and Use Committee of the Institute for Nutritional Sciences, Chinese Academy of Sciences.

2.3. Cell proliferation assay

Cells were seeded in 96-well plates with DMEM containing 5.6 mM glucose and 10% FBS. After cell confluence reached at 60%–80%, the medium was replaced with DMEM containing 5.6 mM glucose and 0.5% FBS. Twenty-four hours later, the cells were cultured in DMEM containing 5.6 or 25 mM glucose, with/without various concentrations of resveratrol or apocynin for 24 h. Cell proliferation was determined by MTT assay (Wang et al., 2008) or by detecting BrdU incorporation using a commercial kit (Cell Proliferation ELISA, BrdU (colorimetric), Millipore, Billerica, MA, USA).

2.4. NADPH oxidase assay

NADPH oxidase activity was measured by the lucigenin chemiluminescence method (Meng et al., 2008). NADPH oxidase activity was defined as relative chemiluminescence (light) units per second per milligram of protein.

2.5. Measurement of intracellular ROS production

The membrane permeable indicator H_2DCF-DA was used to detect intracellular ROS production by rat mesangial cells. The mesangial cells were cultured in DMEM containing 5.6 mM glucose and 0.5% FBS for 24 h. Then the cells were cultured in DMEM containing 5.6 or 25 mM glucose with or without different concentrations of apocynin, N-acetyl-L-cysteine, or resveratrol for 2 h, then were loaded with 10 μmol/l H_2DCF-DA in serum-free DMEM containing 5.6 or 25 mM glucose at 37 °C for 30 min, washed twice with PBS. Intracellular ROS production was detected by the FlexStation II384 fluorometric imaging plate reader (Molecular Devices, Sunnyvale, CA, USA) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

2.6. Western blotting

The cells were lysed with cold lysis buffer and the proteins were electrophoresed on 10% SDS-PAGE gel and transferred onto polyvinylidene difluoride membrane (Wang et al., 2008). The membranes were blocked with 5% nonfat milk and then were incubated with primary antibodies overnight at 4 °C. After incubation with a horseradish peroxidase-conjugated secondary antibody, the protein bands were detected with a Supersignal West Pico chemiluminescent substrate (Pierce, Rockford, IL) and X-Omat BT film (Eastman Kodak Co., Rochester, NY).

2.7. Transient transfection

Rat mesangial cells were grown to 70–80% confluence and transiently transfected with 1 μM phosphorothioate antisense oligonucleotides against $p22^{phox}$ or $p47^{phox}$ (Invitrogen) for 36 h by using Lipofectamine-2000 (Invitrogen, Carlsbad, CA). The expression of $p22^{phox}$, $p47^{phox}$ and fibronectin was quantified by Western blot at indicated times after incubating with DMEM containing 5.6 or 25 mM glucose. The antisense oligonucleotide sequence for $p22^{phox}$ was 5′-GAT CTG CCC CAT GGT GAG GAC C-3′ (Görlach et al.,

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