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 p22phox and p47phox 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.

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 (Kashiara et al., 2010). NADPH oxidase is an important source of ROS production. The phagocyte NADPH oxidase consists of the membrane-associated subunit p22phox and Nox2 (originally named gp91phox), the cytosolic regulatory subunits p47phox, p67phox, p40phox, 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 p22phox, p47phox, p67phox, 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 iodonium decreases renal ROS production and...
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 antioxidantive (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 diabetic 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 (H2DCF-DA) was from Invitrogen (Carlsbad, CA). Antibodies against p22phox and p47phox were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against fibronectin and β-actin were from 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 H2DCF-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 H2DCF-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 p22phox or p47phox (Invitrogen) for 36 h by using LipoFectamine-2000 (Invitrogen, Carlsbad, CA). The expression of p22phox, p47phox 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 p22phox was 5′-GAT CTG CCC CAT GGT GAG ACC C-3′ (Görlich et al.,...
Fig. 1. High glucose induces mesangial cell proliferation through NADPH oxidase mediated ROS production. Rat mesangial cell line (A, B, D and F) or primary rat mesangial cells (C, E and G) were cultured in DMEM containing 5.6 mM glucose (NG) and 0.5% FBS for 24 h, then were cultured in DMEM containing 0.5% FBS and 5.6 (NG) or 25 mM glucose (HG), in the presence or absence of 0.1, 1 mM N-acetyl-l-cysteine (NAC) or 100 mM apocynin (Apo). Cell proliferation were examined after 24 h with MTT assay (A) and BrdU incorporation (B and C), respectively. NADPH oxidase activity was measured after 6 h with lucigenin chemiluminescence method (D and E). ROS production was detected after 2 h with H$_2$DCF-DA (F and G). Results represent the mean ± SD of three independent experiments. *p < 0.05, **p < 0.01, compared with cells cultured in medium containing 5.6 mM glucose. *p < 0.05, **p < 0.01, compared with cells cultured in medium containing 25 mM glucose alone.

2.8. RNA extraction and real-time RT-PCR

Total RNA was extracted from cells using the TRIZOL reagent (Invitrogen, Carlsbad, CA) and depleted of contaminating DNA with Rnase-free Dnase. cDNA was synthesized from 2 μg RNA with M-MuLV reverse transcriptase and random hexamer (Fermentas, Burlington, Ontario, Canada). Quantitative real-time PCR was performed by using an ABI Prism 7500 sequence detector (Applied Biosystems Inc., Foster City, CA). Briefly, reverse-transcribed cDNA in triplicate samples were checked for p22phox and p47phox mRNA levels with SYBR Green PCR master kit (TOYOBO Biotech, Osaka, Japan). PCR primers for target cDNAs were: p22phox: 5′-CAGAAGGTTCCCTAGCAGGAG (sense), and 5′-ACTGAGAAGTTCAGGGCGTT (antisense); p47phox: 5′-AAAGGCGGTCCTATCCCTAC (sense), and 5′-AAACCCAGTCCTGGCCCTTG (antisense). Rat β-actin primers were: 5′-TGGCTCCTAGCACCATGAAG (sense), and 5′-GCCTGTAAGACGGCTGGCTAGA (antisense). Relative levels of target gene mRNA expression were calculated using the $2^{-\Delta\Delta Ct}$ method. Amplification of the target gene cDNA was normalized to β-actin expression.

2.9. Statistical analysis

Data are presented as means ± SD. Statistical differences between groups were analyzed by unpaired Student’s t-test.

3. Results

3.1. High glucose induces mesangial cell proliferation through NADPH oxidase mediated ROS production

We examined the effect of high concentration of glucose on rat mesangial cell proliferation. MTT and BrdU incorporation assays showed that 25 mM glucose significantly enhanced the proliferation of rat mesangial cell line (Fig. 1A and B) and primary rat mesangial cells (Fig. 1C). N-acetyl cysteine (NAC), a ROS inhibitor, and apocynin, an NADPH oxidase inhibitor, both significantly
inhibited high glucose–induced proliferation of rat mesangial cell line (Fig. 1A and B) and rat primary mesangial cells (Fig. 1C) as examined by both MTT and BrdU incorporation assays. As NAC and apocynin at tested concentrations had no effect on viability of mesangial cells (data not shown), these results suggest that ROS and NADPH oxidase mediate high glucose–induced mesangial proliferation. We then checked if high glucose induces ROS production through activating NADPH oxidase. We observed that 25 mM glucose significantly upregulated NADPH oxidase activity and promoted ROS production in rat mesangial cell line which was inhibited by apocynin (Fig. 1D and F). The inhibitory effect of apocynin was confirmed in primary rat mesangial cells (Fig. 1E and G), suggesting that high glucose activates NADPH oxidase which in turn promotes ROS production. All together, the above results suggest that NADPH oxidase activation–derived ROS mediates high glucose–induced mesangial cell proliferation.

### 3.2. High glucose induces mesangial cell proliferation through enhancing NADPH oxidase subunits expression and ROS production

We examined the effect of high glucose on the expression of NADPH oxidase subunits p22phox, p47phox and Nox4 in rat
mesangial cell line and found that while 25 mM glucose had no significant effect on Nox4 expression (data not shown), it induced p22phox and p47phox expression at mRNA and protein levels in a time-dependent manner (Fig. 2A and data not shown). 25 mM glucose upregulated p22phox expression in rat primary mesangial cells at 3 h, which was similar to that in mesangial cell line. High glucose stimulated p47phox expression at 48 h in mesangial cell line but at 6 h after stimulation and sustained up to 48 h in primary cells (Fig. 2B). These results suggest that the primary cells are more sensitive to high glucose than transformed cell line. Transfection of rat mesangial cell line with antisense oligonucleotides against p22phox or p47phox significantly inhibited 25 mM glucose-induced p22phox and p47phox protein expression (Fig. 2C), NADPH oxidase activity elevation (Fig. 2D), and ROS production (Fig. 2F). Inhibition of p22phox and p47phox expression with their respective antisense oligonucleotide in primary rat mesangial cells also inhibited high glucose-enhanced NADPH oxidase activity (Fig. 2E). These results suggest that augmentation of NADPH oxidase activity and promotion of ROS production by high glucose are mediated by upregulation of p22phox and p47phox. Inhibition of p22phox and p47phox expression with their respective antisense oligonucleotide also significantly inhibited 25 mM glucose induced proliferation of rat mesangial cell line (Fig. 2G and H) and primary rat mesangial cells (Fig. 2I). These results suggest that upregulation of p22phox and p47phox expression play a critical role in high glucose-induced mesangial cell proliferation through NADPH oxidase activation and ROS production.

3.3. High glucose stimulates fibronectin expression in mesangial cells through NADPH oxidase subunits upregulation and ROS generation

We examined if NADPH oxidase upregulation and ROS production also contribute to fibronectin upregulation by high glucose in mesangial cells. As shown in Fig. 3A, 25 mM glucose treatment resulted in significant increase in protein level of fibronectin in a time-dependent manner. NAC and apocynin both inhibited fibronectin upregulation by 25 mM glucose but had no effect on the expression of fibronectin in mesangial cells cultured in medium containing 5.6 mM glucose (Fig. 3B), suggesting that ROS and NADPH oxidase mediate high glucose-induced fibronectin expression. Transfection of rat mesangial cell line (Fig. 3C and E) or primary rat mesangial cells (Fig. 3D and F) with antisense oligonucleotides against p22phox or p47phox significantly inhibited fibronectin protein expression. Collectively, our results demonstrated that high glucose induces fibronectin expression in mesangial cells through upregulation of NADPH oxidase subunits p22phox and p47phox and subsequent production of ROS.

3.4. High glucose induces p22phox and p47phox expression through JNK/NF-κB signaling pathway

We further explored the underlying mechanisms of high glucose-induced p22phox and p47phox expression in mesangial cells. Transfection of rat mesangial cell line (Fig. 4A) or rat primary mesangial cells (Fig. 4B) with dominant-negative IκBα plasmids
significantly inhibited high glucose-induced p22phox and p47phox expression, suggesting that high glucose induces p22phox and p47phox expression through activation of NF-κB. We found that JNK inhibitor SP600125 could inhibit high glucose-induced p22phox and p47phox protein expression, while MEK1 inhibitor PD98059 and p38 inhibitor SB203580 had no effect (Fig. 4C). SP600125 could also inhibit p22phox and p47phox protein expression in primary rat mesangial cells (Fig. 4D). Further study showed that 25 mM glucose induced IκBα phosphorylation and degradation in a time-dependent manner (Fig. 4E). SP600125 could inhibit high glucose-induced IκBα phosphorylation and degradation (Fig. 4F). These results suggest that high glucose induces p22phox and p47phox expression through JNK/NF-κB signaling pathway.

3.5. Resveratrol inhibits high glucose-induced mesangial cell proliferation, fibronectin expression and NADPH oxidase-driven oxidative stress

We examined the effect of resveratrol on high glucose-induced mesangial cell proliferation, fibronectin expression, NADPH oxidase activation and ROS production. Treatment of rat mesangial cell line (Fig. 5A and C) or primary rat mesangial cells (Fig. 5B and D) with resveratrol inhibited 25 mM glucose-induced cell proliferation (Fig. 5A and B) and fibronectin expression (Fig. 5C and D) in a dose-dependent manner. Resveratrol inhibited high glucose-induced elevation of NADPH oxidase activity in these cells (Fig. 6A and B), and inhibited high glucose-induced ROS production in a dose-dependent manner (Fig. 6C).

As NADPH oxidase subunits p22phox and p47phox upregulation mediated NADPH oxidase activation and ROS production critically contribute to mesangial cell proliferation and fibronectin expression in response to high glucose, we examined if resveratrol could inhibit high glucose-induced p22phox, p47phox expression. 25 mM glucose significantly induced p22phox and p47phox expression at mRNA and protein levels in rat mesangial cell line (Fig. 6D and E) and primary mesangial cells (Fig. 6F). Resveratrol dose-dependently inhibited high glucose-enhanced expression of p22phox, p47phox (Fig. 6D–F) in these cells. These results suggest that resveratrol attenuated high glucose-induced mesangial cells proliferation and fibronectin expression through inhibiting p22phox and

![Image](https://example.com/image-url)
p47^phox^ upregulation mediated NADPH oxidase activation and ROS production.

3.6. Resveratrol inhibits high glucose-induced JNK phosphorylation and NF-κB activation

Ultimately, we examined the effect of resveratrol on high glucose-induced JNK phosphorylation and NF-κB activation. As shown in Fig. 7A, high glucose increased JNK phosphorylation in rat mesangial cells in a time-dependent manner. Resveratrol inhibited high glucose-induced JNK phosphorylation in a dose-dependent manner in both rat mesangial cell line (Fig. 7B) and rat primary mesangial cells (Fig. 7C). Resveratrol and Sulfasalazine (Sul), an IkBα inhibitor, both inhibited high glucose-induced IkBα phosphorylation and degradation in rat mesangial cell line (Fig. 7D) and primary mesangial cells (Fig. 7E). These results suggest that resveratrol inhibits high glucose-induced JNK phosphorylation and NF-κB activation in mesangial cells.

4. Discussion

In this study, we identified that JNK/NF-κB dependent upregulation of NADPH oxidase subunits, p22^phox^ and p47^phox^, by high glucose resulted in the increase of NADPH oxidase activity and ROS production in renal mesangial cells. NADPH oxidase-derived ROS is necessary for high glucose to induce mesangial cell proliferation and fibronectin expression. These results suggest that high glucose enhances mesangial cell proliferation and fibronectin expression through JNK/NF-κB/NADPH oxidase/ROS pathway. We also demonstrated that resveratrol inhibited mesangial cells from high glucose-induced cell proliferation and fibronectin expression through inhibition of this signaling pathway.

NADPH oxidase is a multicompartmental enzyme. In resting cells, the Nox and p22^phox^, subunits of the NADPH oxidase are located in the cytosol and membranes, p47^phox^, p67^phox^ and p40^phox^ subunits exist in the cytosol as a complex. Upon cell activation, p47^phox^, p67^phox^ and p40^phox^ are phosphorylated and migrate to the membranes to associate with Nox and p22^phox^. The interaction of the different NADPH oxidase subunits allows the formation of an active NADPH oxidase complex. During this process, p22^phox^ serves as a docking site for p47^phox^, p47^phox^ is responsible for transporting the whole cytosolic complex (p47^phox^-p67^phox^-p40^phox^) to the docking site (El-Benna et al., 2009; Nauseef, 2004). Our results showed that knockdown either p22^phox^ or p47^phox^ was sufficient to abolish high glucose-induced NADPH oxidase activation and ROS production, supporting the critical role of p22^phox^ and p47^phox^ in NADPH oxidase assembly and activation. NADPH oxidase is a major source of oxidants in renal cells such as tubular epithelial cells and glomerular mesangial cells. Both in vivo and in vitro studies showed that the upregulation of some NADPH oxidase subunits under diabetic conditions could result in NADPH oxidase activation and ROS production (Lee et al., 2010; Li and Wang, 2010; Park et al., 2011; Sedeek et al., 2010; Shi et al., 2008), consistently, our results showed that high glucose induced NADPH activation and ROS production through upregulating p22^phox^ or p47^phox^ expression in mesangial cells. In addition, high glucose may activate NADPH oxidase through enhancing membrane translocation of its cytosolic subunits (Park et al., 2011).

Increased mesangial cell proliferation and glomerular ECM accumulation are major pathologic features in the early stage of diabetic nephropathy. Studies have indicated that hyperglycemia
plays a central role in the development and progression of the glomerulosclerotic process in diabetes (Kikkawa et al., 2003). Renal NADPH oxidase is an important source of oxidative stress and its expression is enhanced in diabetic nephropathy (Lee et al., 2003). We demonstrated that high glucose induced mesangial cell proliferation through upregulation of NADPH oxidase subunits p22phox and p47phox expression and consequent ROS production. High glucose has been reported to stimulate hepatic stellate cell and vascular smooth muscle cell proliferation through PKC-dependent NADPH oxidase activation and membrane translocation of Rac1, p47phox and p67phox subunits (Sugimoto et al., 2005; Zhu et al., 2010). Therefore, high glucose promotes cell proliferation through regulating NADPH oxidase subunits in cell-specific manner. Furthermore, we demonstrated that upregulation of NADPH oxidase subunits p22phox and p47phox expression and consequent ROS production also play an important role in enhancement of fibronectin expression by high glucose.

In rat diabetic model, NF-κB activation was observed in retina, heart and kidneys (Chen et al., 2003). The activation of NF-κB in human kidney biopsies correlates with severity of proteinuria and degree of glomerulosclerosis in diabetic nephropathy (Mezzano et al., 2004; Schmid et al., 2006). These observations suggest that NF-κB activation may be involved in diabetic nephropathy, but the underlying mechanisms are not clear. Our studies revealed that high glucose induced JNK phosphorylation and its degradation through activation of JNK. Furthermore, we demonstrated that high glucose induced p22phox and p47phox expression through JNK-NF-κB signaling pathway. Supporting our results, high glucose has been reported to activate JNK and NF-κB in mesangial cells (Park et al., 2010; Ha et al., 2002).

Resveratrol is a naturally occurring polyphenol which has been reported to have protective effect against the development of diabetic nephropathy in animals (Palsamy and Subramanian, 2011; Sharma et al., 2006; Chen et al., 2011; Kitada et al., 2011; Tikoo et al., 2008; Ding et al., 2010). Resveratrol attenuates renal hypertrophy and matrix accumulation in diabetic animal models (Sharma et al., 2006; Chen et al., 2011; Kitada et al., 2011; Ding et al., 2010), and inhibits high glucose-induced mesangial cell proliferation in vitro (Ding et al., 2010). In this study, we confirmed that resveratrol could inhibit high glucose-induced mesangial proliferation. Furthermore, we found that resveratrol could inhibit fibronectin expression induced by high glucose. As our results demonstrated that high-glucose induced mesangial cell proliferation and fibronectin expression through JNK/NF-κB/oxidase/ROS signaling pathway, and resveratrol significantly inhibited high-glucose-induced NFκB activation, p22phox and p47phox upregulation, NADPH oxidase activation and ROS production, resveratrol may inhibit high-glucose induced

![Fig. 6. Resveratrol inhibits high glucose-induced NADPH oxidase activation and ROS generation in mesangial cells. Rat mesangial cell line (A, C, D and E) or primary rat mesangial cells (B and F) were cultured in DMEM containing 5.6 mM (NG) or 25 mM glucose (HG) in the presence or absence of different concentrations of resveratrol (RV). NADPH oxidase activity were detected after 6 h with the lucigenin chemiluminescence method (A and B); ROS levels were detected after 2 h with H2DCF-DA (C); the mRNA levels of p22phox and p47phox were detected at 6 h and 12 h, respectively, with real-time RT-PCR (D and E); the protein levels of p22phox and p47phox were detected at 3 h and 48 h, respectively, with Western blot (inserts in D and E; F). Results represent the mean ± SD of three independent experiments. **p < 0.01 compared with cells cultured in medium containing 5.6 mM glucose, ***p < 0.01 compared with cells cultured in medium containing 25 mM glucose alone. Data shown in the inserts in (D) and (E), as well as in (F) are representatives of three independent experiments.](image-url)
mesangial cell proliferation and fibronectin expression through JNK/NF-κB/NADPH oxidase/ROS signaling pathway. Resveratrol has been reported to directly inhibit NADPH oxidase activity and downregulate NADPH oxidase subunit expression (Orallo et al., 2002; Spanier et al., 2009). Therefore, resveratrol may inhibit high-glucose induced mesangial cell proliferation and fibronectin expression through directly inhibition of these molecules. Collectively, our present results provide evidences to explain the molecular mechanisms of the protective effect of resveratrol against diabetic nephropathy.

In summary, we demonstrated that high glucose induced mesangial cell proliferation and fibronectin expression through JNK/NF-κB/NADPH oxidase/ROS signaling pathways. Resveratrol inhibited high-glucose-induced mesangial cell expansion and fibronectin expression through blocking this signaling pathway. Our findings provide new insight into the mechanism of diabetic nephropathy and suggest that interventions targeting this signaling pathway may have the potential to prevent early diabetic nephropathy.

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References