Nox4 is involved in high glucose-induced apoptosis in renal tubular epithelial cells via Notch pathway

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Abstract. It has previously been demonstrated that nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase 4 (Nox4), is important in the pathogenesis of diabetic nephropathy (DN), however the exact mechanisms remain to be elucidated. The present study aimed to examine the effect of Nox4 on the alteration of the Notch pathway and cell apoptosis in the renal tubular epithelial cell line, HKC, under conditions of high glucose (HG; 30 mmol/l glucose). Nox4 and the Notch pathway were inhibited by N-acetylcysteine (NAC), diphenylene iodonium (DPI) or γ-secretase inhibitor (DAPT). The protein levels of Nox4, Notch1, Notch intracellular domain 1 (NICD1), phosphorylated (p) Ras-related C3 botulinum toxin substrate 1 (Rac1), Rac1, B-cell lymphoma 2 apoptosis regulator (Bcl-2), Bcl-2 associated protein X apoptosis regulator (Bax) and cleaved caspase-3 were determined by western blotting. The Nox4 and Notch1 mRNA levels were detected by reverse transcription-quantitative polymerase chain reaction. Intracellular reactive oxygen species (ROS) levels were detected via chloromethyl-2',7'-dichlorodihydrofluorescein diacetate. Apoptotic cells were determined using an Annexin V/propidium iodide apoptosis detection kit. HG upregulated Nox4, Notch1, NICD1, p-Rac1, Bax and cleaved caspase-3 expression levels and downregulated Bcl-2 expression in cultured HKC cells, compared with cells cultured in normal glucose levels. Inhibition of the Notch pathway via DAPT increased Bcl-2 expression, decreased Bax and cleaved caspase-3 levels and prevented HKC cell apoptosis. Inhibition of Nox4 by NAC and DPI inhibited the Notch signaling pathway and ROS generation, which prevented HKC cell apoptosis. These findings indicated that Nox4 potentially mediates HG-induced HKC cell apoptosis via the Notch pathway, and may be involved in renal tubular epithelial cell injury in DN.

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Introduction

Diabetic nephropathy (DN) is a frequently occurring type of progressive kidney disease that develops in the diabetic population. Reactive oxygen species (ROS) have been revealed as important signaling molecules that mediate renal injury in patients with diabetes (1). High glucose (HG) increases intracellular ROS levels in renal cells and contributes to the development and progression of diabetic renal injury (2,3). Under normal physiological conditions, low levels of ROS are produced by the nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase (Nox) family as byproducts of the mitochondrial electron transport chain, and are important in the regulation of various cellular functions including inflammatory gene expression, proliferation and apoptosis (4). The Nox family are membrane bound enzymatic complexes and structural homologues of phagocytic Nox ($gp91^{phox}$/Nox2) and are categorized as follows: Nox1-Nox5 and Dual oxidase Duox proteins 1 and 2 (5). All Nox subunits have been reported to bind ≥1 regulatory subunits, including $p22^{phox}$ which is localized in the membrane, cytosolic submits $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and Ras-related C3 botulinum toxin substrate (Rac) GTPases (5). Various studies have demonstrated that the translocation and binding of $p47^{phox}$ to Nox and the participation of Rac are key steps in the activation of Nox and the generation of ROS (6). Nox4 is a critical ROS-generating complex, expressed in the kidney (5,6).

It has previously been demonstrated that the Notch pathway is involved in the occurrence of DN and various other kidney diseases (7). In mammals, the Notch pathway consists of four receptors (Notch1-4) and five ligands, termed Jagged1, Jagged2, Delta-like ligand (Dll) 1, Dll3 and Dll4. The interaction of these ligands with the Notch receptors results in receptor conformational changes, subsequent γ-secretase mediated proteolysis and release of Notch intracellular domain (NICD), which on transfer to the nucleus, activates gene transcription of Hes family basic helix-loop-helix (BHLH) transcription factor (Hes) 1 and Hes related family BHLH transcription

factor with YRPW motif 1 (8). The Notch signaling pathway is important in differential gene expression and influences cell differentiation, proliferation and apoptosis (9,10).

Yan *et al* (11) demonstrated that transforming growth factor-β (TGF-β) triggers apoptosis in human cultured endothelial cells, an effect dependent on the overexpression of Nox4 and production of ROS via modulation of p38 and Notch pathways. However, it has not yet been revealed whether Nox4 regulates renal tubular epithelial cell apoptosis via the Notch pathway in DN. Previous studies have demonstrated that HG affects normal physical metabolism and function of tubular cells, inducing cell apoptosis (12). In the present study, the HG-induced human renal proximal tubular cell line (HKC) was selected to detect and evaluate the functional activity of Nox4, the Notch signaling pathway and cell apoptotic rate under HG conditions. In addition, Nox4 and the Notch pathway were chemically inhibited in order to further explore the mechanism of tubular cell apoptosis.

Materials and methods

Cell culture. HKC cells were obtained from the cell resource center at Peking Union Medical College (Beijing, China) and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in an environment containing 5% CO_2. HKC cells were grown to 70% confluence and synchronized in serum-free DMEM for 24 h, then stimulated with normal glucose (NG; 5.5 mmol/l D-Glucose; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), HG (30 mmol/l D-Glucose), HG plus γ-secretase inhibitor (DAPT; 1 μmol/l), HG plus N-acetylcysteine (NAC; 5 mmol/l) or HG plus diphenylene iodonium (DPI; 5 μmol/l) all from Sigma-Aldrich; Merck KGaA, at the indicated time points.

Western blotting. The cells were washed with phosphate-buffered saline (PBS) and lysed for 40 min at 4˚C with lysis buffer (20 mmol/l HCl, 2.5 mmol/l EDTA, 10% glycerol, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 10 mmol/l sodium pyrophosphate, 50 mmol/l NaF, 1 mmol/l sodium vanadate, 1 mmol/l phenylmethylsulfonyl fluoride). Subsequently, the homogenate was centrifuged at 14,000 x g for 20 min at 4˚C. The protein concentration was measured using a Bradford assay. Equal amounts of extracted protein samples (40 μg) were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% dry milk for 1 h at 37˚C and incubated with rabbit anti-Nox4 (14347-1-AP; 1:400 dilution; ProteinTech Group, Inc., Chicago, IL, USA), anti-Notch1 (20687-1-AP; 1:1,000 dilution; ProteinTech Group, Inc.), anti-Notch intracellular domain 1 (NICD1; 4147; 1:1,000 dilution; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-phosphorylated (p)-Rac1 (2461; 1:1,000 dilution; Cell Signaling Technology, Inc.), anti-Rac1 (36,742; 1:2,000 dilution; Signalway Antibody LLC, College Park, Maryland, USA), anti-B-cell lymphoma 2 apoptosis regulator (Bcl-2; 4223; 1:1,000 dilution; Cell Signaling Technology, Inc.), anti-cleaved caspase-3 (9664; 1:1,000 dilution; Cell Signaling Technology, Inc.), anti-Bcl-2 associated protein X apoptosis regulator (Bax; 2772; 1:1,000 dilution; Cell Signaling Technology, Inc.) and anti-β-actin (sc-130656; 1:1,000 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) polyclonal antibodies at 4˚C overnight. The membranes were then washed and incubated with a goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (SA00001-2; 1:5,000 dilution; ProteinTech Group, Inc.) for 2 h at room temperature. The labeled bands were quantified using a UVP Image Station Lab works 4.5 (UVP Inc., Upland, CA, USA) and compared with β-actin.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse transcribed using oligo (dT) primer (Sangon Biotech Co., Ltd., Shanghai, China) in the presence of the avian myeloblastosis virus reverse transcriptase (Takara Biotechnology Co., Ltd., Dalian, China), to produce cDNA. cDNA was amplified on the 7900HT Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with SYBR Premix EX Taq™ kit (Takara Biotechnology Co., Ltd.) at default thermal cycling conditions: 2 min at 50˚C, 10 min at 95˚C and then 40 cycles of 15 sec at 95˚C for denaturation and 1 min at 60˚C for annealing and extension. Results were quantified using the relative standard curve method of analysis/ΔC_q method of analysis (13), relative to 18S rRNA. The oligonucleotide primer sequences were as follows: 18S, forward 5'-CGC CGC TAG AGG TGA AAT TC-3' and reverse 5'-CCA GTC GGC ATC GTT TAT GG-3'; Nox4, forward 5'-GTT GGG GCT AGG ATT GTG TCT-3' and reverse 5'-TCG GCA CAT GGG TAA AAG GA-3'; and Notch1, forward 5'-CTA AGA TCT CCT GAG GGC TTC AAA GTG TC-3' and reverse 5'-GCG AAT TCC TTG AAG GCC TCC GGA T-3'.

Intracellular ROS detection. Total ROS levels were detected using the fluorescence probe 5-(and 6) chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate (CM-DCHF-DA; Invitrogen; Thermo Fisher Scientific, Inc.). HKC cells were seeded into 6-well plates at a density of $1x10^6$ cells/ml and incubated under different experimental conditions for 24 h. The cells were washed with PBS three times, trypsinized and centrifuged at 300 x g for 5 min at 4˚C. The cells were then resuspended and incubated in pre-warmed PBS with 10 μM DCHF-DA at 37˚C for 30 min in the dark. Subsequently, cells were washed with PBS three times to remove the free DCFH-DA and fixed with 1% paraformaldehyde for 10 min at 4˚C. The levels of intracellular ROS were quantified using a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) with FlowJo software version 7.6 (FlowJo LLC, Ashland, OR, USA).

Annexin V/propidium iodide (PI) staining assay. Apoptotic rates in the differing groups were detected using an Annexin V/PI staining kit (MultiSciences Biotech Co., Ltd., Hangzhou, China) according to the manufacturer's protocol. HKC cells were incubated under different experimental conditions for 24 h, and then washed with PBS three times, trypsinized and centrifuged at 300 x g for 5 min at 4˚C. Cells were resuspended in 1X binding buffer, incubated with Annexin V-FITC and PI at room temperature for 5 min in the dark. Following fixation with 1% paraformaldehyde for

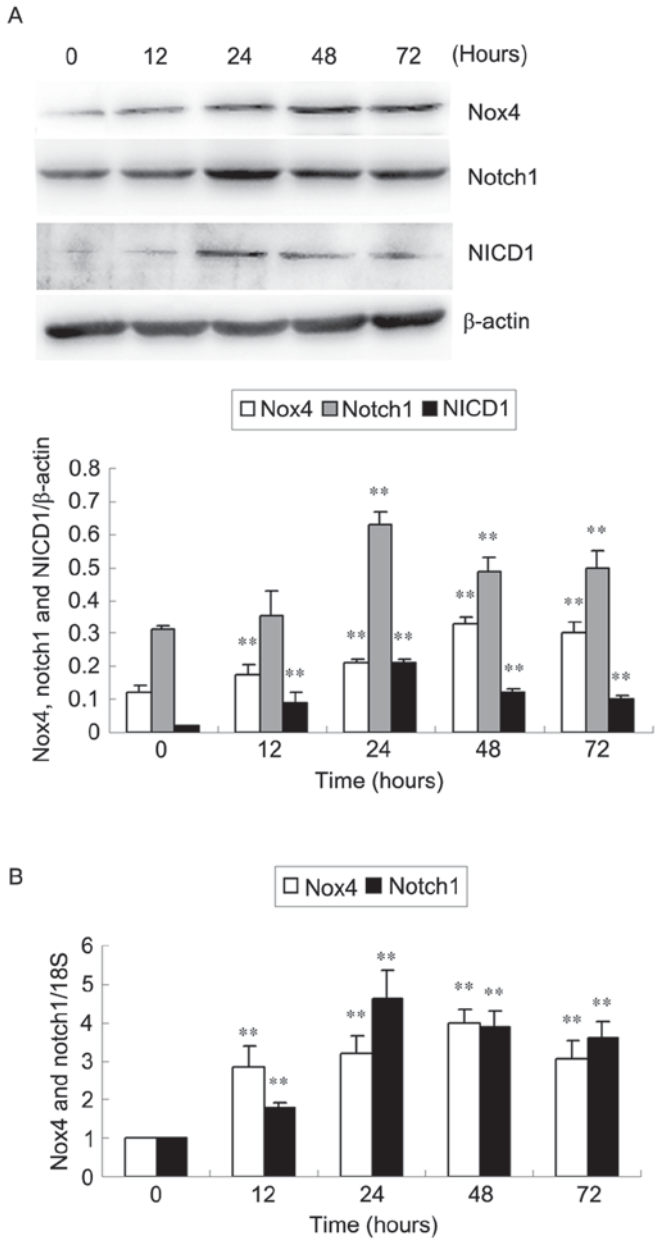

Figure 1. Expression of Nox4, Notch1 and NICD1 in HG-induced HKC cells at the different time points (0-72 h). (A) Representative images and quantification of Nox4, Notch1 and NICD1 protein expression analyzed by western blotting. (B) Nox4 and Notch1 mRNA expression was detected by reverse transcription-quantitative polymerase chain reaction relative to 18S rRNA. Data are expressed as the mean + standard deviation. **P<0.01 vs. control (0 h). Nox 4, nicotinamide adenine dinucleotide phosphate-oxidase oxidase 4; NICD1, Notch intracellular domain 1.

10 min at 4˚C, HKC cells analyzed using a flow cytometer (Epics-XLII; Beckman Coulter, Inc., Brea, CA, USA) with FlowJo software version 7.6 (FlowJo LLC).

Statistical analysis. Data are presented as the mean + standard deviation of at least three independent experiments. All data were analyzed using SPSS software version 15.0 (SPSS, Inc., Chicago, IL, USA). Differences between groups were analyzed using one-way analysis of variance followed by a post hoc Bonferroni's test for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

HG induces Nox4 and Notch pathway expression in HKC cells. Western blotting and RT-qPCR analyses were used to examine HG-induced Nox4 and Notch1 protein and mRNA expression levels in HKC cells (Fig. 1). Nox4 protein and mRNA expression increased as early as 12 h, reached a peak at 48 h and decreased at 72 h (P<0.01; Fig. 1). Notch1 protein and mRNA expression significantly increased at 24 h, then decreased at 48 and 72 h (P<0.01; Fig 1). NICD1 protein expression levels reached a peak at 24 h compared with 0 h following stimulation with HG (P<0.01; Fig. 1A). Furthermore, no significant differences in Nox4, Notch1 and NICD1 expression were observed in HKC cells cultured under NG conditions among different time points (all P>0.05; data not shown).

DAPT inhibits Notch pathway expression and cell apoptosis in HG-induced HKC cells. A significant increase in NICD1 protein expression was observed in HKC cells stimulated with HG for 24 h when compared with NG and this was then decreased with addition of DAPT (P<0.01; Fig. 2A and B). Notch1 protein (Fig. 2A and B) and mRNA (Fig. 2C) expression was also significantly increased in HKC cells stimulated with HG for 24 h compared with NG (P<0.01; Fig. 2A-C), however, DAPT did not inhibit Notch1 protein and mRNA overexpression induced by HG (P>0.05; Fig. 2A-C). HG significantly increased Bax and cleaved caspase-3 protein levels in HKC cells compared with NG, and simultaneously decreased Bcl-2 protein expression (P<0.01; Fig. 2D and E). Treatment with DAPT reversed the alterations in Bax, Bcl-2 and cleaved caspase-3 protein levels in HG-induced HKC cells (P<0.01; Fig. 2D and E). Furthermore, the increased apoptosis rate in HG HKC cells compared with NG cells (P<0.01; Fig. 2F and G) was reduced by treated with DAPT, as demonstrated by flow cytometry (P<0.01 vs. HG; Fig. 2F and G).

NAC and DPI inhibit Nox4 and Notch pathway expression and ROS generation in HG-induced HKC cells. Western blotting was used to examine HG-induced protein expression of Nox4, p-Rac1, Rac1, Notch1 and NICD1 in HKC cells after 24 h (Fig. 3A-C). HG significantly increased Nox4, Notch1 and NICD1 protein expression levels compared with NG (P<0.01; Fig. 3A and B), but this increase was inhibited by NAC and DPI (P<0.05 or P<0.01; Fig. 3A and B). HG significantly increased the ratio of p-Rac1/Rac1 compared with NG (P<0.01; Fig. 3A and C), but while this effect was inhibited by DPI (P<0.01 vs. HG; Fig. 3A and C), no effect was observed with NAC (P>0.05 vs. HG; Fig. 3A and C). No alteration of total Rac1 expression was observed in the cultured HKC cells of different groups (Fig. 3A). RT-qPCR analysis revealed that mRNA expression of Nox4 and Notch1 in the HG-induced HKC cells significantly increased compared with NG (P<0.01; Fig. 3D). Nox4 and Notch1 mRNA levels significantly decreased compared with HG in cells cultured with NAC or DPI in HG culture medium (P<0.05 or P<0.01; Fig. 3D). Intracellular ROS levels were observed to be increased in the HG group compared with the NG group (P<0.01; Fig. 3E), and this HG-induced ROS

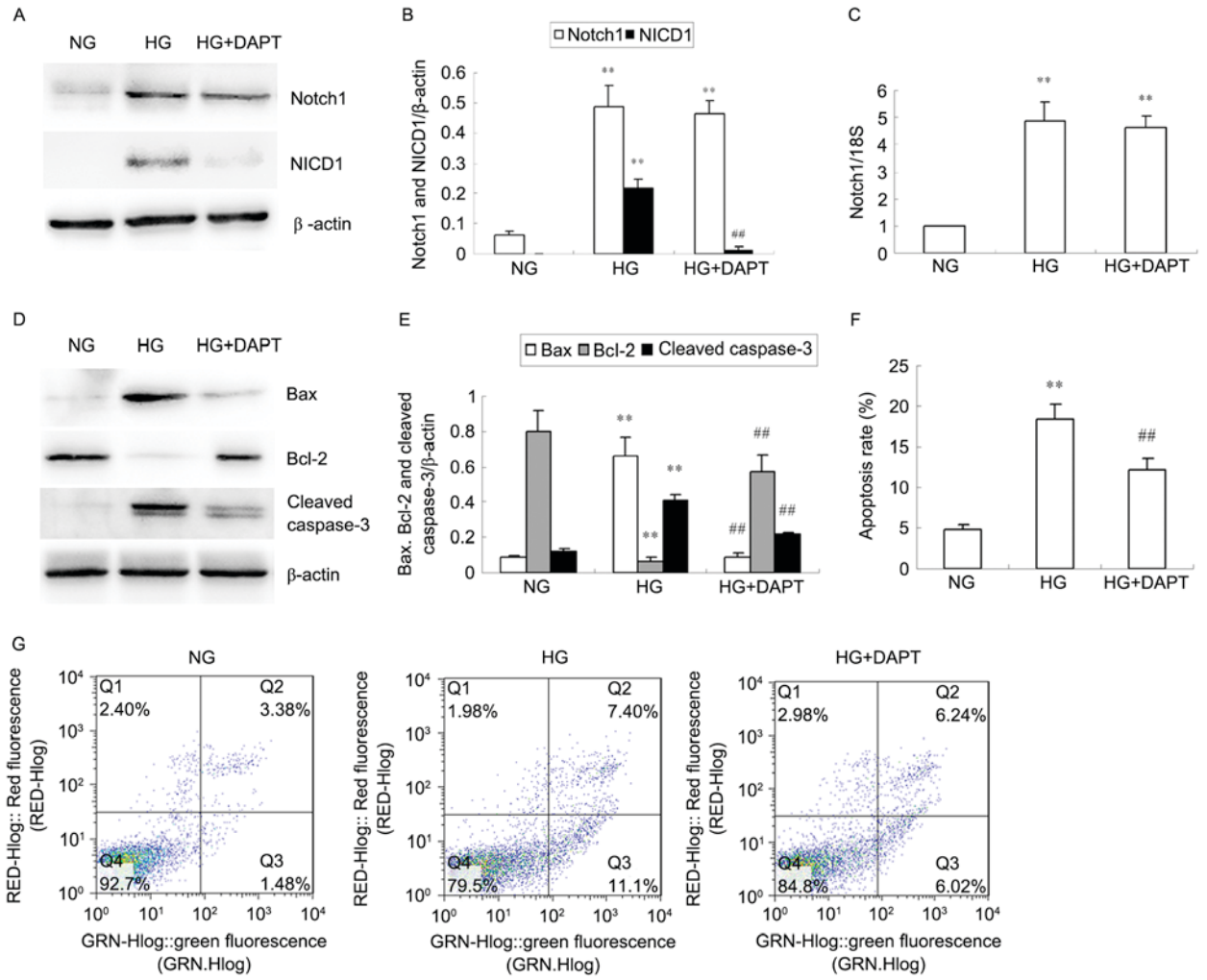

Figure 2. Effects of DAPT on HG-induced Notch pathway expression and HKC cell apoptosis. HKC cells were incubated with NG, HG or HG + DAPT for 24 h. (A) Representative images and (B) quantification of Notch1 and NICD1 protein expression analyzed by western blotting. (C) Notch1 mRNA expression was analyzed by reverse transcription-quantitative polymerase chain reaction relative to 18S rRNA. (D) Representative images and (E) quantification of Bax, Bcl-2 and cleaved caspase-3 protein expression levels analyzed by western blotting. (F) Quantification and (G) representative images and of flow cytometry analysis of HKC cells stained with Annexin V/propidium iodide. Data are expressed as the mean + standard deviation. **P<0.01 vs. NG. $^{\#\#}$P<0.01 vs. HG. NG, normal glucose; HG, high glucose; DAPT, γ-secretase inhibitor; NICD1, Notch intracellular domain 1; Bcl-2, B-cell lymphoma 2 apoptosis regulator; Bax, Bcl-2 associated protein X apoptosis regulator.

production was significantly suppressed by addition of NAC or DPI (P<0.01 vs. HG; Fig. 3E).

NAC and DPI inhibit HG-induced HKC cell apoptosis. The protein levels of Bax and cleaved caspase-3 decreased in HKC cells treated with NAC and DPI compared with levels present in the HG group, whereas Bcl-2 protein levels increased (P<0.05 or P<0.01; Fig. 4A and B). HG-induced HKC cells exhibited a significantly increased apoptosis rate after 24 h compared with the NG cells treated (P<0.01; Fig. 4C and D), whereas NAC and DPI efficiently inhibited HG-induced HKC cell apoptosis (P<0.01 vs. HG; Fig. 4C and D).

Discussion

The results of the present study demonstrated that Nox4 overexpression in HG-induced HKC cells altered Notch pathway levels and induced cell apoptosis. Nox4 and Notch pathway inhibitors prevented HG-induced HKC cell apoptosis.

Nox4 is a constitutively active multisubunit enzyme, which acts as an oxygen sensor and generates various ROS from molecular oxygen using NADPH as the electron donor. Various studies have reported that Nox4 is the major source of ROS in kidney disease including DN, and Nox4-derived ROS are considered to mediate renal hypertrophy, increase myofibroblast activation and renal fibrosis (6,14). Additionally, ROS have been revealed to increase in the presence of HG via activation of NADPH oxidases (15,16). The present study demonstrated that Nox4 protein and mRNA expression increased as early as 12 h and reached a peak at 48 h following stimulation with HG. HG notably increased the levels of Nox4 and Rac1 phosphorylation, which were inhibited by the antioxidant NAC and Nox inhibitor DPI. Hyperglycemia in renal proximal tubule cells may activate Nox and produce superoxide anion and hydrogen peroxide, which may be reversed by treatment with DPI, resulting in an increase in

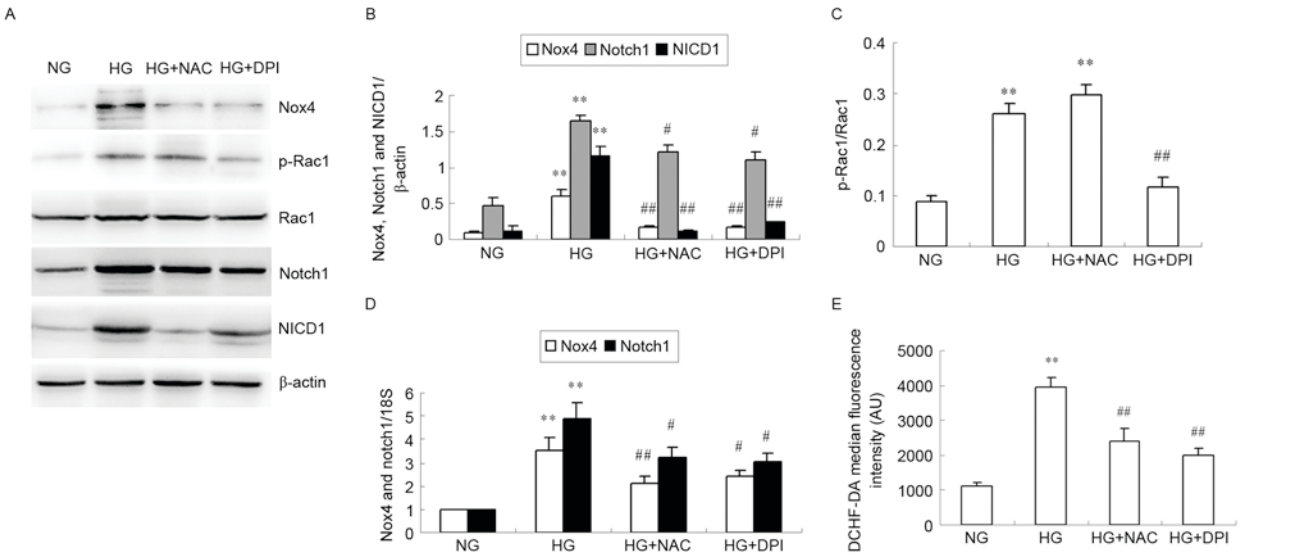

Figure 3. Effects of NAC and DPI on HG-induced Nox4 and Notch pathway expression and ROS generation in HKC cells. HKC cells were incubated with NG, HG, HG + NAC or HG + DPI for 24 h. (A) Representative image of Nox4, p-Rac1, Rac1, Notch1 and NICD1 protein expression levels analyzed by western blotting. Quantification of (B) Nox4, Notch1, NICD1 relative to β-actin and (C) relative p-Rac1/Rac1 expression levels. (D) Nox4 and Notch1 mRNA expression was analyzed by reverse transcription-quantitative polymerase chain reaction relative to 18S rRNA. (E) Quantitative analysis of DCHF-DA fluorescence intensity using flow cytometry. Data were expressed as the mean + standard deviation. **P<0.01 vs. NG. $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. HG. NAC, N-acetylcysteine; DPI, diphenylene iodonium; HG, high glucose; Nox 4, nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase 4; ROS, reactive oxygen species; NG, normal glucose; p, phosphorylated; Rac 1, Ras-related C3 botulinum toxin substrate 1; NICD1, Notch intracellular domain 1; DCHF-DA, dichlorodihydrofluorescein diacetate.

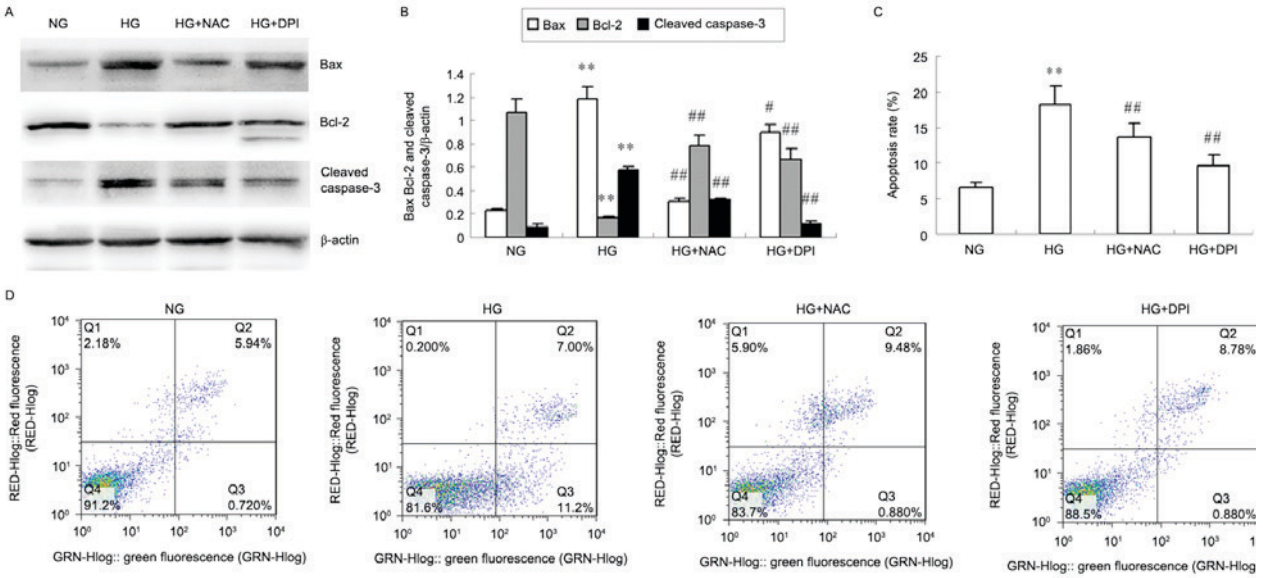

Figure 4. Effects of NAC and DPI on HG-induced HKC cell apoptosis. HKC cells were incubated with NG, HG, HG + NAC or HG + DPI for 24 h. (A) Representative images and (B) quantification of Bax, Bcl-2 and cleaved caspase-3 protein expression levels were analyzed by western blotting relative to β-actin. (C) Quantification and (D) representative images of flow cytometric analysis of HKC cells stained with Annexin V/propidium iodide. Data were expressed as the mean + standard deviation. **P<0.01 vs. NG. $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. HG. NAC, N-acetylcysteine; DPI, diphenylene iodonium; HG, high glucose; NG, normal glucose; Bcl-2, B-cell lymphoma 2 apoptosis regulator; Bax, Bcl-2 associated protein X apoptosis regulator.

TGF-β1 secretion and the activation of the nuclear factor (NF)-κB signal transduction pathway (17). It has additionally been demonstrated that Angiotensin II-induced activation of mitochondrial Nox4 is an important endogenous source of ROS and is associated with cell survival in kidney tubular cells (18).

The Notch pathway is an evolutionarily conserved signaling pathway, which participates in a variety of cellular processes and is important in kidney development (19). Cheng *et al* (20) observed Notch1 activation in the comma-shaped and S-shaped bodies during kidney development. Following inhibition of the Notch pathway, fewer renal epithelial structures are observed, with a severe deficiency in proximal tubules and podocytes, accompanied by an increase in intervening, nonepithelial cells. Notch expression is enhanced in the tubule cells of fibrotic kidneys

from diabetic mice and humans and Notch interacting proteins have been identified that may be pertinent in normal and pathological functioning (21). The present study additionally demonstrated that HG activated the Notch pathway in a time-dependent manner in HKC cells and the maximum expression of Notch1 and NICD1 was observed at 24 h. HG increases Notch1 expression and releases NICD1, which travels into the nucleus and activates the downstream genes in HKC cells (22,23). DAPT, a Notch pathway inhibitor, suppresses activation of the Notch pathway by HG, which may inhibit γ-secretase-mediated proteolytic cleavage of Notch, reducing the release of the NICD from the plasma membrane into the nucleus (10). DAPT inhibited NICD1 expression and did not reduce Notch1 protein and mRNA overexpression in HG-induced HKC cells. DAPT additionally reduced the ratio of Bax/Bcl-2 and cleaved caspase-3 expression in HG-induced HKC cells. Flow cytometry demonstrated that HG induced HKC cell apoptosis, which was subsequently inhibited by treatment with DAPT. These results indicated that HG induced HKC cell apoptosis through activation of the Notch pathway. Notch pathway activity has additionally been revealed to participate in puromycin aminonucleoside-induced renal proximal tubular cell apoptosis via caspase-3 (24). The Notch signaling pathway is important in renal ischemia/reperfusion (I/R), inducing severe tubular damage and resulting in increased NF-κB, Bax and tubular cell apoptosis and reduced Bcl-2 expression (25).

The present study then used the chemical inhibitors NAC and DPI to investigate if Nox4 mediated HKC cell apoptosis via regulation of the Notch pathway in HG medium. HG notably increased levels of Nox4 and Rac1 phosphorylation, which were inhibited by NAC and DPI. Similarly, NAC and DPI inhibited production of ROS and activation of the Notch pathway in HG-induced HKC cells. Treatment with NAC and DPI decreased the ratio of Bax:Bcl-2, reduced cleaved caspase-3 levels and HKC cell apoptosis following HG stimulation. Therefore, HG-induced Nox4 overexpression in HKC cells induced cell apoptosis via activation of the Notch pathway. Similarly, it was previously demonstrated that Rac1 regulates Notch in mediating cerebral I/R-induced production of injurious ROS and cell death *in vitro* and *in vivo* (26). HKC cell apoptosis may lead to a decrease in the number of renal tubular epithelial cells, resulting in renal interstitial fibrosis and the development of DN (27). Inhibition of Nox4 may therefore exhibit the potential to treat DN.

In conclusion, the present results indicated that HG caused the overexpression of Nox4 and Notch signaling molecules in HKC cells. Nox4 upregulation may serve an important role in HG-induced HKC cell apoptosis, through the activation of the Notch pathway. In addition, the present study demonstrated that the blockade of Nox4 using a chemical inhibitor suppressed HG-induced HKC cell apoptosis. Since Nox4 is involved in renal tubular epithelial cell injury in DN, targeting Nox4 may have potential as an alternative therapeutic strategy for the treatment of patients with DN. Further studies are required to explore the molecular mechanisms underlying the involvement of Nox4 in the development of DN, including the relations between Nox4 and other signaling pathways, such as the p38 pathway.

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