NQO1 Alleviates Renal Fibrosis by Inhibiting TLR4/NF- κ B and TGF- β /Smad Signaling Pathway in Diabetic Nephropathy

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Research article

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Abstract

Background: Diabetic nephropathy is one of the main complications of diabetes, inflammation and fibrosis play an important role in its progress. NAD (P) H: quinone oxidoreductase 1 (NQO1) protects cells from oxidative stress and toxic quinone damage. In present study, we aimed to investigate the protective effects and underlying mechanisms of NQO1 on diabetes-induced renal inflammation and fibrosis.

Methods: In vivo, adeno-associated virus serotype 9 was used to infect the kidneys of type 2 diabetes model db/db mice to overexpress NQO1. In vitro, human renal tubular epithelial cells (HK-2) transfected with NQO1 pcDNA were cultured in high glucose. The gene and protein expression were assessed by quantitative real-time PCR, western blot, immunofluorescence, and immunohistochemical staining. Mitochondrial reactive oxygen species was detected by MitoSox red.

Result: Our study revealed that the expression of NQO1 was markedly down-regulated, Toll-like receptor 4 (TLR4) and TGF- β 1 upregulated *in vivo* and *in vitro* under diabetic conditions. Overexpression of NQO1 suppressed pro-inflammatory cytokines secretion (IL-6, TNF- α , MCP-1), extracellular matrix (ECM) accumulation (collagen α 1, Fibronectin) and epithelial-mesenchymal transition (EMT) (α -SMA, E-cadherin) in db/db mice kidney and high glucose cultured human renal tubular cells (HK-2). Furthermore, NQO1 overexpression ameliorated high glucose-induced TLR4/NF- κ B and TGF- β /Smad pathway activation. Mechanistic studies demonstrated that TLR4 inhibitor (TAK-242) suppressed TLR4/NF- κ B signaling pathway, pro-inflammatory cytokines secretion, EMT and ECM-related protein expression in HG-exposed HK-2 cells. In addition, we found that antioxidants NAC and tempol increased the expression of NQO1, decreased the expression of TLR4, TGF- β 1, Nox1, Nox4 and ROS production in HK-2 cells cultured with high glucose.

Conclusions: These above data suggest that NQO1 alleviates diabetes-induced renal inflammation and fibrosis by regulating TLR4/NF- κ B and TGF- β /Smad signaling pathways.

Introduction

Diabetic nephropathy (DN) is one of the most common microvascular complications of diabetes and the main cause of end-stage renal disease (ESRD) in the world. The typical characteristics of diabetic nephropathy are glomerular hyperfiltration, mesangial matrix dilatation, glomerular basement membrane thickening, ECM accumulation, resulting in glomerulosclerosis, tubulointerstitial inflammation, fibrosis, and even renal failure [1]. Renal interstitial fibrosis is an inevitable pathological change in the progression of chronic kidney disease to ESRD. Renal interstitial fibrosis is characterized by dilatation or atrophy of renal tubules, infiltration of interstitial inflammatory cells, proliferation of fibroblasts and increased deposition of interstitial matrix [2]. Inflammatory factors involved in renal fibrosis, the release of local inflammatory factors also promoted the development of renal interstitial fibrosis [3]. Currently, there is no special treatment for renal interstitial fibrosis. Therefore, appropriate treatment must be found to delay the progression of renal interstitial fibrosis.

Epithelial mesenchymal transition (EMT) plays an important role in the development of fibrosis [2]. In the process of EMT, epithelial cells lose their inherent characteristics, which induce the expression of mesenchymal markers and phenotypic changes, resulting in more fibroblast-like cells [4]. Renal tubular fibroblasts are usually induced by myofibroblasts, which are caused by the lack of epithelial marker expression and the activation of interstitial markers [5]. TGF- β 1 is the main factor in promoting fibrosis of chronic nephropathy [6-9]. The activation of TGF- β 1 or its downstream signaling pathway promotes the accumulation of extracellular matrix, leading to basement membrane thickening and glomerulosclerosis [9]. Amygdalin can reduce renal fibrosis in DN model rats by inhibiting TGF- β /Smad signaling pathway to inhibit ECM deposition and EMT [10]. Similar studies have confirmed that dapagliflozin can reduce the expression of TGF- β 1 in the kidney and reduce the EMT of renal tubular epithelial cells, thereby reducing renal interstitial fibrosis in diabetic nephropathy [11].

Toll-like receptors (TLRs) is an important humoral immune regulatory system, which is mainly expressed in immune cells (lymphocytes, dendritic cells, macrophages) and non-immune cells (podocytes, endothelial cells and renal tubular epithelial cells) [12]. TLRs plays an important role in activating innate and adaptive immune responses through the release of several inflammatory cytokine cascades [13]. It is well known that TLR4 upregulation is observed in patients with diabetic nephropathy, and is associated with increased release of various biological mediators such as interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), and monocyte chemoattractant protein 1 (MCP-1) [14,15]. TLR4 has previously been found to cause inflammation-related kidney damage and the development of nephropathy, including diabetic nephropathy [15,16]. Nuclear factor kappa B (NF- κ B) is a downstream effector of the TLR4 signaling pathway and a ubiquitous important nuclear transcription factor that can mediate various inflammatory processes [17]. There is clear evidence that NF- κ B activation plays a crucial role in renal inflammation and fibrosis during the development of diabetic nephropathy [18].

NAD(P)H: quinone oxidoreductase 1 (NQO1) is a cytoplasmic antioxidant flavin protein first discovered in 1958. It increases the level of intracellular NAD⁺ by using NADH as an electron donor to catalyze the reduction of quinones to hydroquinones [19,20]. Recent studies have confirmed that β -lapachone increases the ratio of NAD⁺/NADH by activating NQO1 and alleviates bleomycin-induced pulmonary inflammation and fibrosis in mice and TGF- β 1 induced endothelial cell EMT [21]. In addition, β -lapachone activated NQO1 increased the level of NAD⁺ in cells, inhibited TLR4-mediated inflammatory signal transduction, thus reduced the inflammatory response and cell death in oleomycin-induced acute pancreatitis [22]. However, the role of NQO1 in renal fibrosis in diabetic nephropathy has not been elucidated.

In the current study, *in vivo* experiments we will use type 2 diabetic mice to verify the effect of NQO1 on renal fibrosis in diabetic nephropathy; meanwhile, *in vitro* high glucose cultured HK-2 cells to verify whether NQO1 plays an anti-fibrotic role by regulating the TLR4/NF- κ B and TGF- β /Smad signaling pathway, which provides a new idea for the treatment of diabetic nephropathy.

Materials And Methods

2.1 Antibodies and other reagents

DMEM, DMEM-F12 medium and fetal bovine serum (FBS) was from Gibco (Gaithersburg, Maryland). Penicillin and streptomycin were purchased from Invitgen (Carlsbad, CA). N-acetylcysteine (NAC), mannitol came from Sigma (St. Louis, MO). Tempol and TAK242 came from Selleck Chemicals (Houston, Texas). NQO1 pcDNA3.1(+) plasmid and control pcDNA3.1(+) plasmid were bought from GenePharma (Suzhou, China). FuGENEHD transfection reagents were obtained from Promega (Madison, Wisconsin, USA). TRIzol was bought from Invitrogen (Carlsbad, CA). Takara (Shiga, Japan) was the source of SYBR Premix Ex Taq II. The antibodies against α -smooth muscle actin (α -SMA), E-cadherin, collagen IV, fibronectin, Nox1, Nox4, TLR4, NQO1, Histone H3, IL-6, TNF- α and β -actin were bought from Proteintech (Chicago, IL), while the antibodies of TGF- β 1, NF- κ B p65 and MCP-1 came from Abcam (Cambridge, UK). Antibodies against Smad-2, Smad-3, P-Smad2, P-Smad3 were purchased from Cell Signaling Technology (Beverly, MS). Adeno-associated virus serotype 9 (AAV9) was obtained from HanBio Technology (HH20191024HBYXL-AAV01, Shanghai, China).

2.2 cell culture and transfection

The HK-2 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in DMEM-F12 (3:1) medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 U/mL streptomycin in a 95% air and 5% CO₂ atmosphere at 37 °C. After reaching 40-50% confluence, HK-2 cells were pretreated with serum-free medium for 12 hours, and then transfected with NQO1 pcDNA or control pcDNA plasmid with the help of FuGENE-HD transfection reagent. HK-2 cells were respectively stimulated with normal glucose (NG, 5.6 mmol/L), high glucose (HG, 30 mmol/L), NG + mannitol (M, 24.4 mmol/L), HG + control pcDNA (HG+C), HG + NQO1 pcDNA (HG+NQO1 O/E), HG + Tempol (1 mM), HG + NAC (5 mM) and HG + TAK242 (1 μ M) for 48 hours.

2.3 Animals and treatment

The 8-week-old male C57BL/KsJ db/db mice and littermate control db/m mice (n = 8) were brought from Nanjing University (Nanjing, China). The mice were kept in a temperature-controlled room at 22 $\pm$ 2 °C with a light/dark cycle of 12 hours and standard food and water were free accessed. All experiments were approved by the Animal Ethics Committee of Hebei Medical University. The db/db mice were randomly divided into three groups: db/db group (n = 8), db/db+AAV-Control group (n = 8) and db/db+AAV-NQO1 group (n = 8). To assess the role of NQO1 overexpression on diabetic kidneys, adeno-associated virus serotype 9 was used to treat db/db mice. The renal cortex of db/db mice were injected with 50 μ L 1×10^{11} infective units of AAV-Control or AAV-NQO1 at multiple points in both kidneys. After 12 weeks of treatment, the mice were sacrificed at the age of 20 weeks. Subsequently the renal cortex was collected for detection.

2.4 Western blot analysis

HK-2 cells were washed with PBS for three times and lysed for 40 min at 4 °C with RIPA extraction buffer (Beyotime, China). Furthermore, the frozen kidney tissue protein was extracted following the same procedure. Nuclear and cytoplasmic protein extraction kits (Beyotime, China) were used to extract nuclear and cytoplasmic proteins according to production procedures. Protein concentration was quantified by BCA method (Beyotime, China). The same amount of protein (40 µg) was separated by 10-12% serum dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membrane under 90mV voltage. After blocking with 5% skim milk or BSA at 37 °C for 1 h, the membranes were incubated with the primary antibodies at 4 °C overnight. The next day, the membranes were rinsed three times with TBST (Tris-HCl with Tween-20) and incubated with the respective secondary antibodies for 1 hour at room temperature. After that, the bands were washed 3 times and visualized using an ECL detection reagent. The intensity of each band was semi-quantified with Image J (National Institutes of Health, Bethesda, MD, USA) and was normalized to β-actin.

2.5 RNA Extraction and Quantitative RT-qPCR analysis

Using TRIzol reagent (Invitrogen), total RNA was extracted from kidney tissues or HK-2 cells; and cDNA was prepared using the reverse transcription kit (Promega, Madison, Wisconsin, USA) according to the instructions. The real-time PCR was performed using SYBR Premix Ex Taq II on Agilent Mx3000P qPCR System (Agilent, CA). The sequence of primers used in the experiment is shown in Table 1. The relative expression of the target gene was calculated by $2^{-\Delta\Delta CT}$ method.

2.6 Histological examination

Kidney tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5 µm sections. After deparaffinized and hydrated, the sections were stained with Periodic Acid-Schiff (PAS) and Masson Trichrome, respectively. The degree of glomerular mesangial expansion was assessed in masked manner by PAS staining. The mesangial index was quantified with Image-Pro Plus 6.0 software as (area of mesangial/total area of glomerulus) × 100. The degree of glomerular mesangial expansion was rated on a scale from 0 to 4 as follows: 0, normal; 1, <25%; 2, 25% to 50%; 3, 50%-75%; 4 > 75%. Collagen deposition was further determined by Masson trichrome staining in masked manner. For Masson trichrome, the blue collagen deposition was taken as a positive signal, and the percentage of the positive area in the entire visual. Images were observed by the microscope (Olympus BX63; Tokyo, Japan) to exam glomerular, tubular morphology and collagen deposition. Quantitative analysis of Masson staining was performed using Image J (National Institutes of Health, Bethesda, MD, USA).

2.7 immunohistochemistry

Immunohistochemistry (IHC) was used to detect the protein expression levels in renal tissues. Renal tissues were fixed in 4% paraformaldehyde overnight. Then the kidneys were embedded in paraffin and 4 µm sections were prepared. The sections were subjected to dewaxing and rehydration. Subsequently, antigen retrieval at 121°C for 15 min using sodium citrate buffer (pH 6.0). After natural cooling, three percent hydrogen peroxide inactivation for 20 min. Afterwards, renal tissue sections were blocked with 1%

BSA in phosphate-buffer saline (PBS) for 30 min and then were incubated with primary antibodies against α -SMA, E-cadherin, collagen α 1(I), Fibronectin, TLR4, NQO1, MCP-1, IL-6, TNF- α , F4/80, TGF- β 1 and NF- κ B p65 separately overnight at 4 °C. Next, after PBS washing, the slides were incubated with an HRP-conjugated secondary antibody for 30 min at 37 °C. After PBS washing, goat anti-rabbit IgG secondary antibody was added to sections incubated at 37 °C for 1 h. Then renal tissues were observed after 3,3'-diaminobenzidine-tetrahydrochloride-dihydrate (DAB) staining. Finally, the slides were observed using an inverted microscope (Olympus BX63; Tokyo, Japan). Image Pro Plus 6.0 was used to quantify the integrated optical density (IOD) of positive areas.

2.8 Immunofluorescence Staining

The expressions of α -SMA, E-cadherin, collagen α 1(I), Fibronectin, TLR4, NQO1, Nox1, Nox4, TGF- β 1 and NF- κ B p65 in the HK-2 cells were evaluated by immunofluorescence. Briefly, HK-2 cells were washed with PBS three times, then fixed with 4% formaldehyde at room temperature for 40 min, and subsequently incubated for 5 min in room temperature in 0.2% Triton X-100. The cells blocked with PBS containing 5% bovine serum at room temperature for 1 h and incubated with the primary antibody at 4°C overnight. The Next day HK-2 cells washed with PBS three times and incubated with the goat anti-rabbit IgG (H+L) fluorescent secondary antibody (1:200) for 2 h. After washing with PBS three times, HK-2 cells were incubated for 10 min in 40,60-diamidino-2-phenylindole (DAPI) to stain the nuclei, fluorescence images were captured with laser confocal microscope.

2.9 ROS detection

Mitochondrial reactive oxygen species was detected by MitoSox red (Invitgen). Cells were cultured in six-well chamber for 48 hours and subsequently incubated with MitoSox red in a final concentration of 5 μ M in the dark for 30 min at 37 °C. Then the cells were washed for three times by HBSS (Hank's balanced salt solution) and images were acquired using a confocal microscope (Leica, Germany). The mitochondrial reactive oxygen species intensity was quantified using the software Image-Pro Plus 6.0.

2.10 Statistical analysis

The data were expressed as mean $\pm$ SD. Student's t-test was used for the analysis of two groups and one-way ANOVA was applied for the analysis of no less than three groups. When $P < 0.05$, the results were defined as statistically significant.

Results

3.1 NQO1 overexpression prevented renal interstitial fibrosis in db/db mice.

To evaluate the role of NQO1 overexpression in renal interstitial fibrosis in diabetic nephropathy, adeno-associated virus serotype 9 was used to treat db/db mice (Figure 1A). The renal tissue was histologically examined by PAS and Masson staining. As shown in Figure 1B, compared with the db/m group, db/db group had severer glomerular mesangial expansion and tubulointerstitial collagen deposition.

Furthermore, the kidney histopathologic lesions were less severe after treatment with AAV-NQO1 as observed in db/db mice. As shown in Figure 1C, D Western blot and RT-qPCR analysis indicated that db/db mice exhibited NQO1 expression declined and the renal fibrosis markers Fibronectin and collagen IV expression increased compared with db/m mice. However, those changes of NQO1, Fibronectin and collagen IV expression were reversed by AAV-NQO1 treatment. Consistent with the protein expression data, immunohistochemical staining showed that the expression of NQO1 decreased, while the expression of Fibronectin and collagen IV increased in the db/db group, these changes were significantly remitted in db/db+AAV-NQO1 group (Figure 1E). These results suggest that NQO1 plays an important role in the induction of fibrosis in diabetic kidney.

3.2 NQO1 overexpression inhibited the TGF- β /Smad signaling pathway and the expression of E-cadherin and α -SMA in db/db mice kidney.

TGF- β /Smad is the classic pathway of fibrosis. To explore the effect of NQO1 overexpression on the TGF- β /Smad signaling pathway in the kidney of diabetic mice, Western blot, RT-qPCR and immunohistochemical staining were performed. As Figure 2A-D shown, the TGF- β 1, p-Smad2 and p-Smad3 protein levels were increased in the kidney of db/db mice. The renal TGF- β 1, p-Smad2 and p-Smad3 protein levels were decreased in db/db+AAV-NQO1 group compared with the db/db group. The mRNA level of TGF- β 1 was consistent with the protein expression level (Figure 2E). Immunohistochemical staining showed that there were increased positive-stained areas of TGF- β 1 in kidney sections of db/db mice; After AAV-NQO1 treatment, the renal TGF- β 1 expression levels of db/db mice were effectively decreased (Figure 2H). Epithelial marker E-cadherin and mesenchymal marker α -SMA were detected to evaluate the effect of NQO1 overexpression on renal cell EMT. Western blot and RT-qPCR results showed that E-cadherin expression in the kidney of db/db mice was lower than that of db/m mice, while α -SMA expression was higher than that of db/m mice; and the trends were reversed by AAV-NQO1 treatment (Figure 2A, F, G). Furthermore, immunohistochemistry data showed similar results as Western blot (Figure 2H).

3.3 NQO1 overexpression suppressed pro-inflammatory cytokines expression in db/db mice.

Renal interstitial fibrosis is initially characterized by interstitial inflammation, which eventually leads to extensive fibrotic changes [23]. To investigate the role of NQO1 overexpression on inflammation in diabetic kidney, the production of pro-inflammatory cytokines IL-6, MCP-1 and TNF- α were detected. The results of Western blot and RT-qPCR showed that compared with the db/m group, the production of renal IL-6, TNF- α and MCP-1 was clearly increased in the db/db mice. However, NQO1 overexpression inhibited diabetes-induced IL-6, TNF- α and MCP-1 production (Figure 3A-B). In addition, we detected the expression of F4/80 (a macrophage marker) in the kidney. Compared with db/m mice, the number of F4/80-positive macrophages was increased in the kidney of db/db mice; After AAV-NQO1 treatment, the increase of F4/80-positive cells in the kidney of db/db mice was significantly reduced (Figure 3C). At the same time, the immunohistochemical staining results of IL-6, TNF- α and MCP-1 verified the Western blot and RT-qPCR results (Figure 3C).

3.4 NQO1 overexpression reduced TLR4 and nuclear NF- κ B p65 expression in the kidney of db/db mice.

In order to explore the anti-inflammatory mechanism of NQO1, the effect of NQO1 overexpression on diabetes-induced activation of the TLR4/NF- κ B signaling pathway was examined. As illustrated in Figure 4A-B protein and mRNA expression levels of TLR4 were up-regulated in the db/db group. However, after treatment with AAV-NQO1, the protein and mRNA expression levels of TLR4 in the db/db group were reduced. NF- κ B is a key transcriptional factor involved in the inflammatory process, and the nuclear level of NF- κ B p65 is an indicator of NF- κ B signaling pathway activation. The level of nuclear NF- κ B p65 protein in the db/db group was significantly higher than that in the db/m group, while AAV-NQO1 treatment significantly reduced the increase in nuclear NF- κ B p65 protein expression (Figure 4C). The immunohistochemical results of TLR4 and NF- κ B p65 further confirmed the results of Western blot analysis (Figure 4D).

3.5 NQO1 overexpression blocked HG-induced ECM related proteins expression in HK-2 cells.

To determine the effect of high glucose on NQO1 expression, HK-2 cells were treated with 30 mM glucose for 0, 6, 12, 24, 48, and 72 hours. As shown in Figure 5A, the expression of NQO1 significantly decreased in time-dependent manner in HK-2 cells treated with HG. Extracellular matrix accumulation plays an important role in the occurrence and development of interstitial fibrosis. We further explored the effect of transfection with NQO1 pcDNA plasmid on ECM related proteins in HG-cultured HK-2 cells. As shown in Figure 5B-C, NQO1, collagen IV and Fibronectin protein and mRNA were detected. Compared with NG group, the expression of NQO1 was decreased, while the expression of Fibronectin and collagen IV increased in HK-2 cells treated with HG. Transfection with NQO1 pcDNA plasmid up-regulated NQO1 expression and reduced collagen IV and Fibronectin in HG-exposed HK-2 cells. The results of immunofluorescence staining of NQO1, collagen IV and fibronectin were consistent with those of Western blot analysis (Figure 5D).

3.6 NQO1 overexpression inhibited the activation of TGF- β /Smad signaling pathway and EMT in HK-2 cells under HG ambience.

To investigate the role of NQO1 overexpression on TGF- β /Smad signalling pathway in HG-treated HK-2 cells, we performed Western blot analysis on TGF- β 1, p-Smad2 and p-Smad3. The results of Figure 6A-D revealed that NQO1 pcDNA transfection decreased TGF- β 1, p-Smad2 and p-Smad3 protein expression in HG-treated HK-2 cells. As indicated by RT-qPCR, overexpression of NQO1 reversed the high expression of TGF- β 1 induced by high glucose in HK2 cells (Figure 6E). In addition, the immunofluorescence results showed that NQO1 overexpression blocked the high expression of TGF- β 1 in HG-exposed HK2 cells (Figure 6H). EMT plays an important part in the occurrence and development of interstitial fibrosis. We used Western blot, RT-qPCR and immunofluorescence method to detect the expression of E-cadherin and α -SMA. Figure 6A, F, G-H showed that the expression of E-cadherin was down-regulated and the expression of α -SMA was up-regulated in HK-2 cells treated with HG. However, transfection with NQO1 pcDNA plasmid attenuated the decrease of E-cadherin expression and the increase of α -SMA expression in HG-cultured HK-2 cells. In addition, HK-2 cells exhibited a typical epithelial cuboidal shape in NG group,

but HG treatment produced a spindle-like shape. However, this HG-induced morphological change was reversed by NQO1 pcDNA transfection (Figure 6G).

3.7 NQO1 overexpression restrained pro-inflammatory cytokines expression and TLR4/NF- κ B signaling pathway in HG-cultured HK-2 cells.

Pro-inflammatory cytokines were detected by Western blot and RT-qPCR in HG-exposed HK-2 cells. The expression of IL-6, TNF- α and MCP-1 in the HG group was higher than that in the NG group. Transfection with the NQO1 pcDNA plasmid can reduce the expression levels of these cytokines (Figure 6A-B). To explore the effects of NQO1 overexpression on the TLR4/NF- κ B signalling pathway activation in HG-treated HK-2 cells, we performed Western blot analysis and immunofluorescent staining of TLR4 and nuclear NF- κ B p65 protein expression. As shown in Figure 6C, E-F, the expression levels of TLR4 and nuclear NF- κ B p65 proteins were significantly higher in the HG group than in the NG group; Transfection with NQO1 pcDNA plasmid decreased HG-induced TLR4 and nuclear NF- κ B p65 expression levels in HK-2 cells. In addition, the mRNA level of TLR4 in HG group was higher than that in NG group, and the high expression of TLR4 mRNA was reversed by NQO1 pcDNA transfection in HK-2 cells cultured with high glucose (Figure 6D). In conclusion, NQO1 may reduce the secretion of pro-inflammatory cytokines by inhibiting the TLR4/NF- κ B signaling pathway.

3.8 TLR4 inhibitor (TAK-242) suppressed TLR4/NF- κ B signaling pathway, pro-inflammatory cytokines secretion, EMT and ECM-related protein expression in HG-exposed HK-2 cells.

TAK-242 is a new TLR4 inhibitor that used to explore the underlying mechanism NQO1 involved in inflammation and fibrosis in HK-2 cells under HG conditions. As exhibited by western blot, the high expression of TLR4 and nuclear NF- κ B p65 induced by high glucose could be significantly inhibited by TAK242 or NQO1 pcDNA treatment in HK-2 cells (Figure 8A-B, F). Then the pro-inflammatory cytokines including IL-6, TNF- α and MCP-1 were surely blocked by TAK-242 or NQO1 pcDNA transfection in HG-cultured HK-2 cells (Figure 8A, C). In addition, the high expression of Fibronectin, collagen IV and α -SMA was suppressed, and the low expression of E-cadherin was up-regulated by NQO1 pcDNA or TAK242 treatment in HG-exposed HK-2 cells (Figure 8A, D-E). Taken together, these results uncovered that NQO1 overexpression ameliorated DN by inhibiting pro-inflammatory cytokines inflammation and fibrosis via down-regulating TLR4/NF- κ B signaling pathway in HG-cultured HK-2 cells.

3.9 Effect of antioxidant Tempol or N-acetylcysteine (NAC) on NQO1, TLR4, TGF- β 1, Nox1 and Nox4 expression and ROS generation in HG-cultured HK-2 cells

Antioxidant Tempol or NAC were utilized to explore their roles on HG-induced NQO1, TLR4, TGF- β 1, Nox1 and Nox4 expression in HK-2 cells. Western blot results implicated that NQO1 protein was down-regulated under HG conditions, while TLR4, TGF- β 1, Nox1 and Nox4 proteins were up-regulated. However, the high protein levels of NQO1, TLR4, TGF- β 1, Nox1 and Nox4 were reversed by NQO1 pcDNA, Tempol and NAC treatment in HK-2 cells under HG conditions (Figure 9A-D). Besides, immunofluorescence detection of NQO1, TLR4 and TGF- β 1 confirmed the results of the Western blot (Figure 9E). As shown in Figure 9E-F,

after transfected with NQO1 pcDNA or Tempol and NAC treatment, the level of ROS in mitochondria induced by HG decreased significantly in HG-exposed HK-2 cells. These results suggested that NQO1 may block the expression of TLR4 and TGF- β 1 by oxidation-dependent.

Discussion

Diabetic nephropathy is one of the most serious complications of diabetes, and it is also the main cause of ESRD. Tubulointerstitial fibrosis is a key process in the development of diabetic nephropathy to ESRD [24]. It is well known that renal inflammation, extracellular matrix deposition and EMT play an important role in the occurrence and development of diabetic nephropathy, which eventually lead to renal fibrosis. NQO1 is a homodimeric flavoprotein, under oxidative stress, the expression of NQO1 is triggered by the Keap1/NRF2/ARE pathway. Studies have shown that the expression of NRF2 and its downstream gene NQO1 is reduced in diabetes-induced kidney damage [25]. In addition, Geum-LanHong et al. have shown that NQO1 gene deletion aggravates glomerular changes and interstitial fibrosis in STZ-induced diabetic mice [26]. In this present study, we found that the expression of NQO1 in the kidneys of diabetic mice was reduced and collagen deposition increased, while overexpression of NQO1 alleviated diabetes-induced renal fibrosis. Taken together it is suggested that NQO1 is a promising target to ameliorate diabetic nephropathy via regulating renal fibrosis.

The increase of renal collagen, ECM accumulation and EMT are the main factors of nephropathy, and they are also important signs of the development of diabetic nephropathy into renal fibrosis. Our findings show that overexpression of NQO1 significantly decreased the levels of Fibronectin, collagen IV and α -SMA expression, increased the expression of E-cadherin in the kidney of diabetic mice and HK-2 cells cultured with high glucose. Similar results were reported in BML-111 promoted the activation of Nrf2/HO-1/NQO1 attenuated expression of ECM proteins fibronectin and collagen IV [27]. In line with this, Katikireddy KR et al. reported that Menadione-induced EMT is potentiated by the absence of NQO1 in postmitotic corneal cells [28]. In summary, NQO1 can improve renal fibrosis by regulating ECM and EMT, but its underlying mechanism has not yet been elucidated.

TGF- β 1 is a strong fibrogenic cell factor, TGF- β /Smad signaling pathway can mediate the development of EMT and ECM accumulation, and promote the development of renal fibrosis [29-31]. In current study, our results revealed that overexpression of NQO1 significantly inhibited the expression of TGF- β 1 and the level of Smad2/3 phosphorylation. Similarly, Jia Q et al. found that Genistein exhibited reno-protective effects in diabetic rats by activating the Nrf2/HO-1/NQO1 pathway, and suppressed the TGF- β 1/Smad3 pathway to alleviated renal fibrosis [32]. Next, we explored the possible mechanism by which NQO1 regulates the TGF- β /Smad signaling pathway. Previous studies have shown that NADPH oxidase is the major source of reactive oxygen species (ROS) production in diabetic nephropathy [33]. Furthermore, excessive ROS activate the expression of TGF- β 1, which contributes to EMT and ECM accumulation as a key characteristic in renal fibrosis [34, 35]. Our results suggested that transfection of NQO1 pcDNA, antioxidant Tempol or NAC treated up-regulated the expression of NQO1, down-regulated the expression of TGF- β 1, Nox1 and Nox4, and reduced the production of ROS in HG exposed HK-2 cells. In line with this,

Huang K et al. show that USP9X promoted Nrf2/ARE/NQO1 pathway activation to prevent the production of ROS and blocks the TGF- β /Smad signaling pathway, eventually alleviated the pathological process of diabetic renal fibrosis [36]. These results suggest that NQO1 overexpression reduces the production of ROS, further inhibits high glucose-induced TGF- β /Smad signal pathway activation and reduces the accumulation of ECM and EMT, therefore alleviates diabetic renal fibrosis.

Chronic inflammation plays an important role in the progression of DN [37]. In present study, we tested the expression of pro-inflammatory cytokines. The results suggested that the protein expression levels of IL-6, TNF- α and MCP-1 in the kidney of diabetic mice were significantly higher than those of normal mice. Similarly, HK-2 cells stimulated by high glucose secreted more IL-6, TNF- α and MCP-1 than normal glucose cells. However, overexpression of NQO1 effectively reduced the level of these inflammatory cytokines in kidney of diabetic mice and high glucose treated HK-2 cells. Reportedly, Nootkatone significantly increased the expression of NQO1 and blocked pro-inflammatory cytokine TNF- α , IL-1 β , and IL-10 upregulation by regulating the AMPK signaling pathway, thereby improving the neuroinflammation induced by LPS [38]. As well, the expression of NQO1 increased after mycobacteria infection, while knocking down NQO1 increased the differentiation of macrophages, the activation of NF- κ B and the secretion of pro-inflammatory cytokines TNF- α and IL-1 β after infection [39]. These results suggest that NQO1 could inhibit the secretion of pro-inflammatory cytokines and its protective effect on diabetic renal injury may be related to its anti-inflammatory effect.

The correlation between inflammation and fibrosis has been confirmed and supported by morphological evidence. Previous studies have shown that inflammation is necessary in the process of renal interstitial fibrosis [40]. Studies have reported that high glucose directly activates TLR4, in podocytes and renal tubular epithelial cells leading to inflammation and interstitial fibrosis [41]. TLR4, as the promoter of the signal pathway, activates NF- κ B to increase the production of pro-inflammatory cytokines. Previous studies have shown that the expression of IL-1 β , IL-6 and TNF- α is partly dependent on NF- κ B [42]. Our studies have shown that overexpression of NQO1 or TLR4 inhibitor TAK-242 treatment can reduce the expression of TLR4 and nuclear NF- κ B p65, inhibit the production of pro-inflammatory cytokines in HG-cultured HK-2 cells. Recently reported NQO1 could inhibit the TLR-dependent production of selective cytokines such as IL-6, IL-12 and GM-CSF on LPS stimulated macrophages [43]. Moreover, we found antioxidant Temol or NAC treated up-regulated the expression of NQO1, down-regulated the expression of TLR4 in HG exposed HK-2 cells. Similar to our findings, the antioxidants NAC and glutathione inhibited mechanical stress induced TLR2 and TLR4 expression, ROS production and pro-inflammatory cytokines in periodontal ligament cells [44]. Therefore, these findings indicate that HG-induced TLR4 activation and pro-inflammatory cytokine production may be oxidation-dependent, and NQO1 improve renal interstitial fibrosis might by inhibiting the TLR4/NF- κ B signal pathway and the secretion of pro-inflammatory cytokines.

To sum up, the results show that overexpression of NQO1 improves renal inflammation, EMT and ECM accumulation in diabetic mice. In addition, we also found that overexpression of NQO1 could inhibit the activation of TLR4/NF- κ B and TGF- β /Smad signaling pathway in diabetic mice kidney and high glucose

cultured HK-2 cells, while inhibition of TLR4 could reduce NF- κ B activation and pro-inflammatory cytokines production in high glucose cultured HK-2 cells. These findings suggest that NQO1 may improve the renal interstitial fibrosis of diabetic nephropathy by inhibiting TLR4/NF- κ B and TGF- β /Smad signaling pathway (Figure 10).

Declarations

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Author Contributions

Duojun Qiu: Conceptualization, Methodology, Investigation, Writing - original draft. Shan Song: Formal analysis, Writing - review & editing. Yuhang Wang: Data curation. Yawei Bian: Investigation. Ming Wu: Data curation, Writing - review & editing. Haijiang Wu: Writing - review & editing, Funding acquisition. Yonghong Shi: Conceptualization, Project administration. Huijun Duan: Conceptualization, Funding acquisition.

Availability of data and materials

All data generated or analyzed during this study are included in this article.

Ethics approval and consent to participate

All experiments were approved by the Animal Ethics Committee of Hebei Medical University.

Consent for publication

Authors agreed to this publication.

Competing interests

The authors declare no conflicts of interest.

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Tables

Table 1 The sequences of the PCR primer pairs used in this study.

NQO1 (human)	F: ATGTATGACAAAGGACCCTTCC
	R: TCCCTTGCAGAGAGTACATGG
TLR4 (human)	F: GTCTCCTCCACATCCTCCCT
	R: CTCCCAGAACCAAACGATG
TGF- β 1 (human)	F: GACCGCAACAACGCAATCTATGAC
	R: TGCTCCACAGTTGACTTGAATCTCTG
Fibronectin (human)	F: ACAAGCATGTCTCTCTGCCA
	R: CCAGGGTGATGCTTGGAGAA
Collagen IV (human)	F: GACCGAGCCCTACAAAACCC
	R: CTCAGGCTCTCAGGCCAC
E-cadherin (human)	F: ATGCTGATGCCCCCAATACC
	R: ATCTTGCCAGGTCCTTTGCT
α -SMA (human)	F: CTTGTTTGGGAAGCAAGTGGG
	R: GATTCCTGACAGTGCTTGGC
IL-6 (human)	F: GCCAGAGCTGTGCAGATGAGT
	R: TGGCATTGTGGTTGGGTCAG
TNF- α (human)	F: GGCAGTCAGATCATCTTCTCGA
	R: CGGTTCAGCCACTGGAGCT
MCP-1 (human)	F: ACGCTTCTGGGCCTGTTGTTCA
	R: TGGGGCATTAACTGCATCTGGCT
GAPDH (human)	F: CTGACTTCAACAGCGACACC
	R: TGCTGTAGCCAAATTCGTTGT
NQO1 (mouse)	F: AGGATGGGAGGTA CT CGAATC
	R: TGCTAGAGATGACTCGGAAGG
TLR4 (mouse)	F: GGGCCTAAACCCAGTCTGTTTG
	R: GCCCGGTAAGGTCCATGCTA
TGF- β 1 (mouse)	F: GCAACAATTCCTGGCGTTACCTTG
	R: CAGCCACTGCCGTACAACTCC
Fibronectin (mouse)	F: GATGTCCGAACAGCTATTTACCA

	R: CCTTGCGACTTCAGCCACT
Collagen IV (mouse)	F: GTCGTGGAGACATCGG
	R: CCTGCCTCACCCCTTT
E-cadherin (mouse)	F: CAGCCTTCTTTTCGGAAGACT
	R: GGTAGACAGCTCCCTATGACTG
α -SMA (mouse)	F: CCTTCGTGACTACTGCCGAG
	R: GTCAGCAATGCCTGGGTACAT
IL-6 (mouse)	F: AAAGAGTTGTGCAATGGCAATTCT
	R: AAGTGCATCATCGTTGTTCATACA
TNF- α (mouse)	F: ACTGAACTTCGGGGTGATTG
	R: GCTTGGTGGTTTGCTACGAC
MCP-1 (mouse)	F: TTGACCCGTAAATCTGAAGCTAAT
	R: TCACAGTCCGAGTCACACTAGTTCAC
GAPDH (mouse)	F: CGGAGTCAACGGATTTGGTCGTAT
	R: AGCCTTCTCCATGGTGGTGAAGAC

Figures

Figure 1

NQO1 overexpression prevented renal interstitial fibrosis in db/db mice. (A) The db/db mice in db/db+AAV-Control group (n = 8) and db/db+AAV-NQO1 group (n = 8) were renally injected with 50 μ L 1×10^{11} infective units of AAV-Control or AAV-NQO1 at multiple points in both kidneys. 12 weeks later, the mice were sacrificed. (B) Kidney sections were stained with Periodic Acid-Schiff (PAS) and Masson trichrome (Masson) (scale bar, 50 μ m, n = 9), mesangial expansion and fibrotic area were measured. (C-E)

The effect of AAV-NQO1 on the expression of NQO1, Fibronectin and collagen IV in db/db mice was detected by Western blot (n = 6) RT-qPCR (n = 6) and immunohistochemistry staining (Scale bar, 50 μ m, n = 9). Values are expressed as mean $\pm$ SD. **P < 0.01 versus db/m group; ##P < 0.01 versus db/db+AAV Control group.

Figure 2

NQO1 overexpression inhibited the TGF- β /Smad signaling pathway and renal α -SMA and E-cadherin expression in db/db mice. (A-D,F) The expression of TGF- β 1, Smad-2, Smad-3, P-Smad2, P-Smad3, α -SMA and E-cadherin was evaluated by Western blot (n = 6). (E,G) The mRNA levels of TGF- β 1, α -SMA and E-cadherin were detected by RT-qPCR (n = 6). (H) Immunohistochemical staining of kidney sections with TGF- β 1, α -SMA and E-cadherin antibody in diabetic mice (Scale bar, 50 μ m, n = 9). Values are expressed as mean $\pm$ SD. **P < 0.01 versus db/m group; ##P < 0.01 versus db/db+AAV Control group.

Figure 3

NQO1 overexpression suppressed pro-inflammatory cytokines expression in db/db mice. (A-B) The expression of IL-6, TNF- α and MCP-1 was detected by western blot and RT-qPCR (n = 6). (C) Immunohistochemical staining of F4/80, IL-6, TNF- α and MCP-1 in kidney sections (Scale bar, 50 μ m, n = 9). Values are expressed as mean $\pm$ SD. **P < 0.01 versus db/m group; ##P < 0.01 versus db/db+AAV Control group.

Figure 4

NQO1 overexpression reduced TLR4 and nuclear NF-κB p65 expression in the kidney of db/db mice. (A-B) The expression level of TLR4 was detected by the Western blot and RT-qPCR (n = 6). (C) The expression level of nuclear NF-κB p65 protein was detected by the Western blot (n = 6). (D) Renal TLR4 and NF-κB p65 were detected by immunohistochemical staining in db/db mice (Scale bar, 50μm, n = 9). Values are expressed as mean±SD. **P < 0.01 versus db/m group; ##P < 0.01 versus db/db+AAV Control group.

Figure 5

NQO1 overexpression blocked HG-induced Fibronectin and collagen IV expression in HK-2 cells. (A) The protein level of NQO1 was analyzed by western blot in HK-2 cells treated with 30 mM glucose for 0, 6, 12, 24, 48, and 72 h (n = 3). (B,C) The expression of NQO1, Fibronectin and collagen IV was detected by the Western blot and RT-qPCR (n = 3). (D) The expression levels of NQO1, Fibronectin and collagen IV were detected by immunofluorescence (Scale bar, 10 μ m, n = 6). Values are expressed as mean $\pm$ SD. *P < 0.05, **P < 0.01 versus 0h or NG group; ###P < 0.01 versus HG+C group.

Figure 6

NQO1 overexpression inhibited the activation of the TGF-β/Smad signaling pathway and EMT in HK-2 cells under HG ambience. (A-D,F) The protein levels of TGF-β1, Smad-2, Smad-3, P-Smad2, P-Smad3, α-SMA and E-cadherin were analyzed by western blot (n = 3). (E,G) The mRNA levels of TGF-β1, α-SMA and E-cadherin were detected by RT-qPCR (n = 3). (H) The expression levels of TGF-β1, α-SMA and E-cadherin were detected by immunofluorescence (Scale bar, 10 μm, n = 6). (G) Morphological changes of HK-2 cells

were analysed under an inverted microscope (Scale bar, 50 μ m). Values are expressed as mean $\pm$ SD. **P < 0.01 versus NG group; #P < 0.05, ##P < 0.01 versus HG+C group.

Figure 7

NQO1 overexpression restrained TLR4/NF- κ B signaling pathway in HG-cultured HK-2 cells. (A,B) The expression of IL-6, TNF- α and MCP-1 was analyzed by Western blot and RT-qPCR (n = 3). (C,D) The expression of TLR4 was analyzed by western blot and RT-qPCR (n = 3). (E) The expression of nuclear NF- κ B p65 was analyzed by western blot (n = 3). (F) The expression levels of TLR4 and NF- κ B p65 were detected by immunofluorescence (Scale bar, 10 μ m, n = 6). Values are expressed as mean $\pm$ SD. **P < 0.01 versus HG+C group; #P < 0.05, ##P < 0.01 versus HG+NQO1 O/E group;

Figure 8

TLR4 inhibitor (TAK-242) suppressed TLR4/NF-κB signaling pathway, pro-inflammatory cytokines secretion, EMT and ECM-related protein expression in HG-exposed HK-2 cells. (A-E) The protein levels of TLR4, IL-6, TNF-α, MCP-1, Fibronectin, collagen IV, α-SMA and E-cadherin were analyzed by western blot (n = 3). (F) The protein level of nuclear NF-κB p65 was analyzed by western blot (n = 3). Values are expressed as mean±SD. **P < 0.01 versus HG group.

Figure 9

Effect of antioxidant Tempol or NAC on NQO1, TLR4, TGF-β1, Nox1 and Nox4 expression and ROS generation in HG-cultured HK-2 cells. (A-D) The protein levels of NQO1, TLR4, TGF-β1, Nox1 and Nox4 were analyzed by western blot (n = 3). (E) The protein expression levels of NQO1, TLR4 and TGF-β1 were detected by immunofluorescence (Scale bar, 50 μm, n = 6). (E) Mitochondrial ROS was assessed by the

fluorescence probe MitoSOX Red (Scale bar, 50 μ m, n = 6). (F) The quantitative analysis of mitochondrial ROS. Values are expressed as mean $\pm$ SD. **P < 0.01 versus HG group.

Figure 10

Model of the effect of NQO1 improve the renal interstitial fibrosis of diabetic nephropathy by inhibiting TLR4/NF- κ B and TGF- β /Smad signaling pathway.