Heliyon 8 (2022) e10515

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

tBHQ attenuates podocyte injury in diabetic nephropathy by inhibiting NADPH oxidase-derived ROS generation via the Nrf2/HO-1 signalling pathway

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ARTICLE INFO

Keywords:
Nrf2
tBHQ
ROS
Podocytes
Diabetic nephropathy

ABSTRACT

Aims: Oxidative stress plays a crucial role in podocyte injury in diabetic nephropathy (DN). tert-Butylhydroquinone (tBHQ) is an activator of Nrf2 that exerts protective effects in diabetic mice, but the underlying mechanism of tBHQ in the podocytes of DN is not fully understood.

Materials and methods: A high glucose (HG)-induced HK2 cell model and streptozotocin-induced rat model of DN were established and treated with tBHQ or apocynin. The expression levels of Nrf2, HO-1, NOX2 and NOX4 were determined by Western blot or immunohistochemical staining. The level of oxidative stress in podocytes or kidney tissues was assessed using DCFH-DA or dihydroethidium (DHE) staining. Cell injury was assessed by F-actin staining and flow cytometry analysis.

Key findings: We showed that HG treatment increased the expressions of NOX2 and NOX4 and enhanced ROS production in podocytes. Inhibition of NADPH oxidase activity by apocynin dramatically attenuated HG-induced ROS production and further alleviated cell injury and apoptosis in podocytes. Moreover, we found that HG inhibited the Nrf2/HO-1 signalling pathway in podocytes; however, tBHQ treatment significantly activated the Nrf2 signalling pathway, inhibited NADPH oxidase activity, and attenuated ROS production and cell injury in HG-treated podocytes. Furthermore, we observed that tBHQ treatment partially attenuated renal injury, activated the Nrf2 signalling pathway, inhibited NADPH oxidase activity and reduced ROS generation in the kidneys of STZ-induced diabetic rats.

Significance: These results suggest that tBHQ exerts a protective role in hyperglycaemia-induced podocyte injury, and that the potential protective mechanism of tBHQ involves inhibiting NADPH oxidase-derived ROS generation by activating the Nrf2/HO-1 signalling pathway.

1. Introduction

Diabetic nephropathy (DN) is a serious complication of diabetes mellitus and is a leading cause of end-stage renal disease [1]. Podocytes are terminally differentiated epithelial cells and play a pivotal role in maintaining the function of the glomerular filtration barrier, damage to which is associated with proteinuria [2]. Previous studies have revealed that podocyte damage and loss are closely related to the occurrence and development of DN [3]. However, the potential pathogenesis mechanism of DN has not been fully explained.

Accumulating evidence has demonstrated that oxidative stress plays a crucial role in DN progression and has come to be recognized as the major mechanism underlying the pathogenesis of DN [4]. NADPH oxidase (NOX) is the major source of reactive oxygen species (ROS), and the upregulation or hyperactivation of various NADPH oxidase isozymes contributes to renal oxidative stress, which causes damage to kidney tissues under metabolic conditions such as hyperlipidaemia, hyperglycaemia and hyperuricaemia [5]. The NOX family consists of seven members: NOX1–5 and the dual oxidases, Duox1-2 [6]. It is now clear that NADPH oxidase catalytic subunits such as Nox1, Nox2, Nox4 and

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https://doi.org/10.1016/j.heliyon.2022.e10515
Received 11 December 2021; Received in revised form 3 March 2022; Accepted 26 August 2022

Nox5 are abundantly expressed in kidney tissues [7, 8]. Previous studies have revealed that NADPH oxidase-derived ROS overproduction is involved in high glucose-induced podocyte injury [9], and upregulated NADPH oxidase activity was also found in the glomeruli in a rat model of streptozotocin-induced diabetic nephropathy [10].

Nuclear factor E2-related factor-2 (Nrf2) is a critical transcription factor that regulates the cellular antioxidant response [11]. Under normal conditions, Keap1 interacts with Nrf2 in the cytoplasm to mediate Nrf2 proteasomal degradation. Under stimulated conditions, Nrf2 dissociates from Keap1 and translocates into the nucleus; Nrf2 then binds with antioxidant response elements (AREs) to transcribe downstream antioxidant genes such as hemeoxygenase-1 (HO-1) [12]. The Nrf2/HO-1 signalling pathway plays a crucial role in protecting cells from oxidative stress damage. Han et al. found that activation of the Nrf2 antioxidant pathway protects mouse kidney function from ischaemia–reperfusion injury [13]. Additionally, Chen et al. showed that activation of the Nrf2 signalling pathway alleviated cisplatin-induced nephrotoxicity by eliminating excessive ROS production [14]. Tertiary butylhydroquinone (tBHQ), an activator of Nrf2, has been reported to possess antioxidant function and play a protective role in METH-induced neurotoxicity [15], ischaemia/reperfusion (I/R)-induced hepatic injury [16] and doxorubicin-induced cardiotoxicity [17]. Recently, Zhou et al. found that tBHQ ameliorated contrast-induced nephropathy in rats by activating the Nrf2/Sirt3/SOD2 signalling pathway [18]. However, the exact role and underlying mechanism of tBHQ in the podocytes of DN are still not fully elucidated.

Thus, our study aimed to investigate the effects of tBHQ on HG-induced podocyte injury using in vitro and in vivo models and to further explore the possible mechanism involved in the modulation of the Nrf2/HO-1 pathways and NADPH oxidase by tBHQ.

2. Materials and methods

2.1. Cell culture

Conditionally immortalized mouse podocytes were obtained from American Type Culture Collection (Manassas, CA, USA). Podocytes were cultivated in RPMI-1640 medium supplemented with 10% foetal bovine serum (FBS, Gibco), 100 μg/mL streptomycin, 1000 U/L penicillin and 10 U/ml recombinant IFN-γ at 33 °C for proliferation. To induce differentiation, the cells were cultured without IFN-γ at 37 °C for 10–14 d. For subsequent experiments, the differentiated cells were treated with normal glucose (NG, 5.5 mM D-glucose), high glucose (HG, 30 mM D-glucose), or a hypertonic solution (5.5 mM glucose + 24.5 mM D-mannitol) for 24 h.

2.2. Animals

Male Sprague–Dawley (SD) rats (200–220 g) were obtained from the Animal Centre of Chengdu University of Traditional Chinese Medicine, housed in a temperature-controlled room (24 ± 1 °C) under a 12 h light/dark cycle and allowed free access to food and water. All animal experiments were carried out with the approval of the Ethics Committee of Chengdu University of Traditional Chinese Medicine. Following acclimatization for two weeks, the rats were randomly divided into a control group and a diabetic group. Diabetes was induced in the rats by intraperitoneal injection of freshly prepared streptozotocin (STZ, Sigma, dissolved in 0.1 M citrate buffer at pH 4.5) at a dose of 60 mg/kg for 5 consecutive days. The control rats were injected with the same amount of sodium citrate buffer alone. The rats with blood glucose levels ≥16.7 mmol/L were regarded as being diabetic rats, and they were randomly separated into 2 groups: the DN group (n = 8) and the DN + tBHQ group (n = 8). The diabetic rats in the DN + tBHQ group received an intraperitoneal injection of tert-butylhydroquinone (tBHQ) (Sigma–Aldrich, 50 mg/kg) every other day for 10 weeks. At the end of the experiment, all animals were weighed and sacrificed, and the blood and kidney tissues were harvested for further analysis.

2.3. Measurement of ROS generation

Intracellular ROS generation was measured with the ROS-sensitive fluorescent probe DCFH-DA according to the manufacturer's protocol. After the treatment, podocytes were incubated with 10 μM MDCFH-DA in serum-free culture medium at 37 °C in the dark for 20 min. Cells were washed gently with PBS to remove the free DCFH-DA probe, and the images were observed with fluorescence microscopy. The level of oxidative stress in kidney tissues was assessed using dihydroethidium (DHE). Four-micrometre-thick frozen kidney sections were incubated with 10 μM DHE probe at 37 °C in the dark for 20 min. After three washes with PBS, kidney tissue sections were observed under a fluorescence microscope.

2.4. Transmission electron microscope (TEM) examination

Kidney tissues (1 mm^3) were fixed in 2% glutaraldehyde for 12 h at 4 °C and then postfixed in 1% osmium tetroxide for 2 h. The fixed sections were dehydrated in alcohol, embedded in pure araldite and sliced with an ultramicrotome. Finally, the sections were stained with uranyl acetate and lead citrate and then examined with a transmission electron microscope (Hitachi, Tokyo, Japan).

2.5. Immunohistochemistry (IHC) staining

The paraffin-embedded kidney sections were dewaxed and subjected to antigen retrieval. After inactivating endogenous peroxidase activity and blocking with goat serum solution for 20 min at room temperature, kidney sections were incubated at 4 °C overnight with the following primary antibodies: rabbit anti-Nrf2 (1:200, CST), rabbit anti-NOX2 (1:200, Abcam), and rabbit anti-NOX4 (1:200, Abcam). Subsequently, kidney sections were incubated with biotin-conjugated secondary antibody and then incubated with a diaminobenzidine (DAB) solution followed by staining with haematoxylin.

2.6. Immunofluorescence staining

The cells were grown on glass coverslips and fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.1% Triton X-100 and blocked with 5% bovine serum albumin (BSA) at room temperature. The cells were incubated with rabbit anti-podocin antibody (1:200, Abcam) and rabbit anti-Nrf2 antibody (1:200, CST) overnight at 4 °C and then incubated with the appropriate FITC-labelled secondary antibody. After washing with PBS, the cells were stained with DAPI for 5 min, and then images were acquired with a fluorescence microscope.

2.7. F-actin staining

The cells were grown on glass coverslips. After treatment, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and blocked with 5% BSA. Subsequently, the cells were stained with Alexa 594-phalloidin (Invitrogen) at 37 °C in the dark for 30 min. After washing with PBS, the cells were counterstained with DAPI for 5 min, and then images were observed with a fluorescence microscope.

2.8. Apoptosis assay

Podocyte apoptosis was assessed with an Annexin V-FITC Apoptosis Detection Kit (BD Pharmingen, USA) according to the manufacturer's instructions. Briefly, the treated cells were incubated with annexin V binding buffer followed by PI staining in the dark and were analysed immediately using flow cytometry. Early apoptotic cells (annexin V positive/PI negative) and late apoptotic cells (annexin V positive/PI positive) were defined as apoptotic cells.

2.9. Western blot analysis

Kidney tissues and podocytes were lysed with cold RIPA lysis buffer (BestBio, Shanghai, China) on ice and then sonicated and centrifuged at 12,000 g for 10 min at 4 °C. Protein concentrations were determined with a BCA Protein Assay Kit. Protein samples were loaded and separated using SDS–PAGE and then electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Massachusetts, USA). After blocking with 5% nonfat milk, the PVDF membranes were incubated at 4 °C overnight with the following primary antibodies: rabbit anti-Nrf2 (1:1000, CST), rabbit anti–HO–1 (1:1000, Abcam), rabbit anti-NOX2 (1:200, Abcam), rabbit anti-NOX4 (1:200, Abcam), rabbit anti-Bax (1:1000, CST), rabbit anti-Bcl-2 (1:1000, CST), rabbit anti-Cleaved-caspase3 (1:1000, CST), rabbit anti-Lamin B (1:1000, Santa Cruz) and mouse anti-β-actin (1:5000, Sungene Biotech). After incubation with appropriate horseradish peroxidase-conjugated secondary antibodies (Solarbio, Beijing, China), the immunoreactive bands were visualized using an ECL detection kit (Pierce, Rockford, IL, USA).

2.10. Measurement of NADPH oxidase activity

Podocyte NADPH oxidase activity was determined using an NADPH oxidase activity assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. The absorbance was read with a spectrophotometer at 340 nm. The values of NADPH oxidase activity are presented as relative fold changes of control samples.

2.11. Statistical analysis

Quantitative data are expressed as the mean ± SEM of at least three independent experiments. Comparisons of more than two groups were performed using a one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. GraphPad Prism software was used for performing statistical analyses. A $P < 0.05$ was considered statistically significant.

3. Results

3.1. HG conditions increased the expressions of NOX2 and NOX4 and enhanced ROS production in podocytes

NADPH oxidase is a major source of ROS generation, which plays an essential role in mediating cell oxidative stress [19]. Our Western blot analysis revealed that the expressions of NADPH oxidase subunits, including Nox2 and NOX4, was increased in a time-dependent manner in podocytes exposed to HG (Figure 1A, 1B and 1C). Moreover, we also found that cytosolic ROS generation was notably increased in podocytes treated with HG, as indicated by staining with the ROS-sensitive fluorescent probe DCFH-DA (Figure 1D and 1E).

3.2. Inhibition of NADPH oxidase activity with apocynin attenuated HG-induced ROS production and cell injury in podocytes

To elucidate whether NADPH oxidase is associated with ROS generation and cell injury, we used a NADPH oxidase inhibitor (apocynin) to pretreat podocytes. As shown in Figure 2A and 2B, we found that apocynin dramatically decreased intracellular ROS production and inhibited NADPH oxidase activity in HG-treated podocytes. Moreover, using immunofluorescence staining, we examined the protein expression of podocin, an important slit diaphragm protein of the podocyte foot process. We found that HG significantly decreased the expression of podocin; however, apocynin treatment upregulated the expression of podocin (Figure 2C). We next observed the changes in the cytoskeleton using fluorescein isothiocyanate (FITC)-conjugated phalloidin staining. As indicated in Figure 2E, our immunofluorescence results showed that HG clearly induced cytoskeletal damage, which manifested as disrupted and

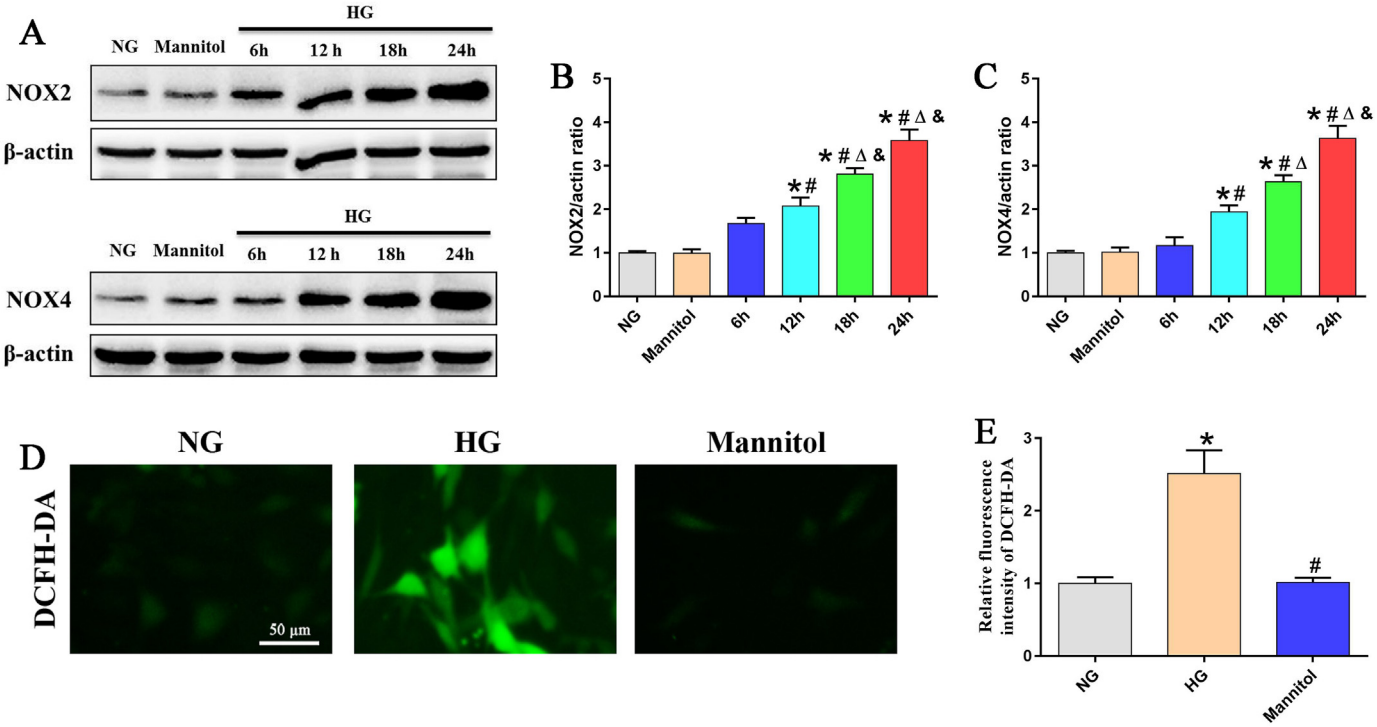

Figure 1. HG conditions increased the expressions of NOX2 and NOX4 and enhanced ROS production in podocytes. (A) Western blot analysis of NOX2 and NOX4 expression in podocytes after treatment with HG at different time points. (B–C) Quantification analysis of NOX2 and NOX4. *$P < 0.05$ vs. NG group, $^{\#}P < 0.05$ vs. mannitol group, $^{\triangle}P < 0.05$ vs. 6 h group, and $^{\&}P < 0.05$ vs. 12 h group. (D) Podocytes were treated with HG for 24 h, and intracellular ROS production was measured with DCFH-DA staining. (E) Quantitative analysis of the relative fluorescence intensity of DCFH-DA. *$P < 0.05$ vs. NG group, $^{\#}P < 0.05$ vs. HG group. The uncropped images of (A) were referred to in supplementary Figure 1.

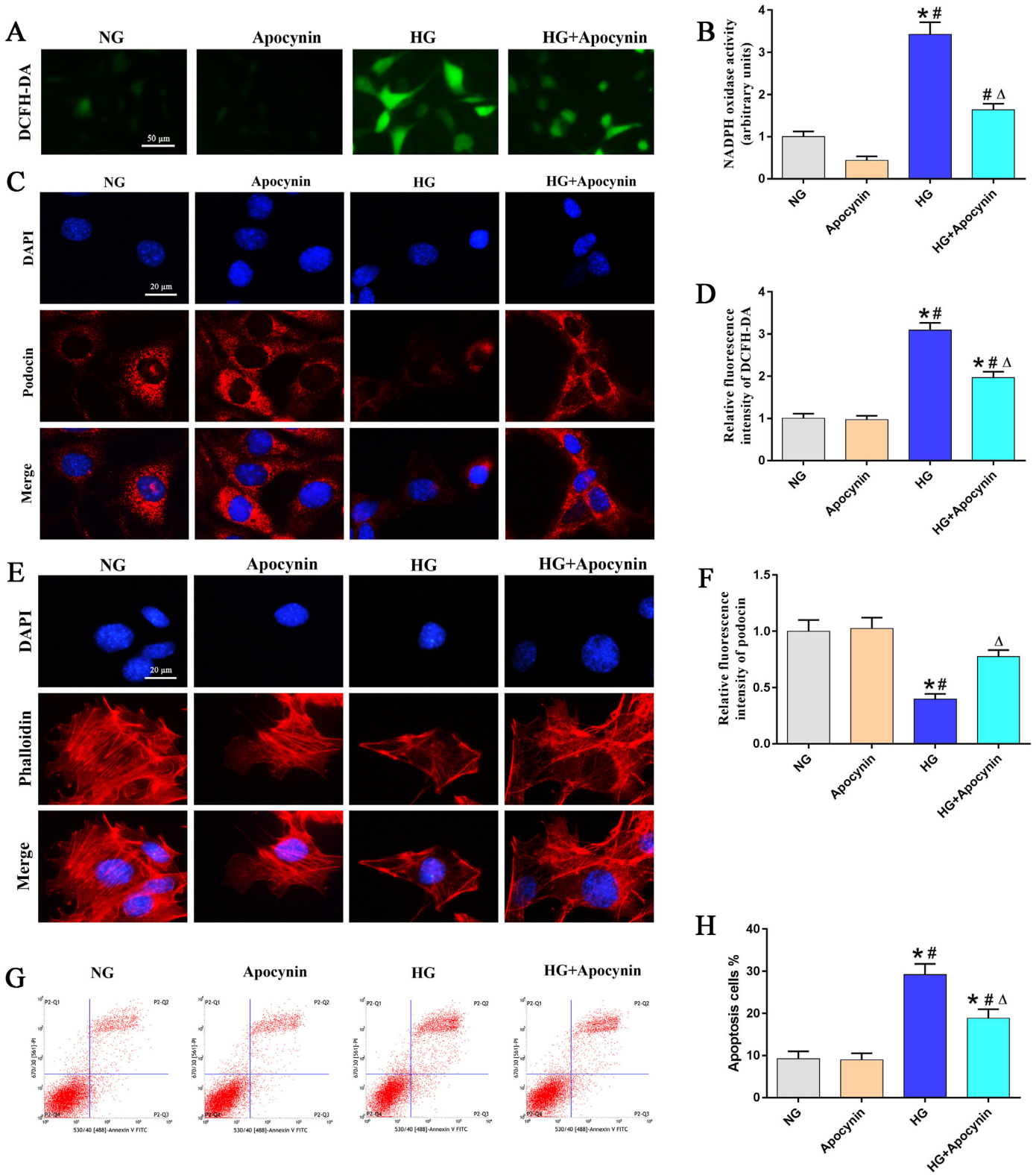

Figure 2. Inhibition of NADPH oxidase activity with apocynin attenuated HG-induced ROS production and cell injury in podocytes. (A) Podocytes were pretreated with apocynin (100 μM)) for 2 h and then treated with or without HG for 24 h. Intracellular ROS production was measured with DCFH-DA staining. (B) NADPH oxidase activity was determined with an NADPH oxidase activity assay kit. (C) Immunofluorescence analysis of podocin expression in podocytes in different groups. (D) Quantitative analysis of the relative fluorescence intensity of DCFH-DA. (E) Phalloidin staining was used to label the cytoskeleton of podocytes in different groups. (F) Quantitative analysis of the relative fluorescence intensity of podocin. (G) Flow cytometry analysis of podocyte apoptosis under different conditions. (H) Quantitative analysis of apoptotic cells in Panel G. (*P $<$ 0.05 vs. NG group, $^{\#}$P $<$ 0.05 vs. apocynin group, and $^{\triangle}$P $<$ 0.05 vs. HG group).

decreased actin stress fibres in podocytes. However, apocynin treatment protected against HG-induced cytoskeletal damage. Furthermore, we revealed the role of apocynin in HG-induced apoptosis of podocytes. As shown in Figure 2G and 2H, apocynin treatment significantly suppressed HG-induced podocyte apoptosis as assessed by flow cytometry analysis. These results demonstrate that NADPH oxidase-derived ROS mediates HG-induced podocyte apoptosis.

3.3. HG conditions inhibited the Nrf2/HO-1 signalling pathway in podocytes

To determine the effect of HG on the Nrf2/HO-1 signalling pathway in podocytes, the expressions of Nrf2 and HO-1 were detected by Western blot analysis. As shown in Figure 3A, 3B and 3C, the expressions of Nrf2 and HO-1 were markedly decreased in podocytes treated with HG (30 mM) for 24 h. In addition, our immunoblot analyses revealed that mannitol had no influence on Nrf2 and HO-1 expressions compared with normal culture conditions. Furthermore, a time-dependent reduction in Nrf2 and HO-1 protein levels was also observed in podocytes under HG conditions (0, 6, 12, 18, and 24 h) (Figure 3D, 3E and 3F).

3.4. Activation of the Nrf2 signalling pathway with tBHQ inhibited NADPH oxidase activity and attenuated ROS production in HG-treated podocytes

To investigate the possibility that the Nrf2 signalling pathway might regulate NADPH oxidase activity and ROS production, we used an inducer of Nrf2, tertiary butylhydroquinone (tBHQ), to activate the Nrf2 signalling pathway. First, HK-2 cells were pretreated with tBHQ at progressive concentrations of 10 μM, 20 μM, 30 μM and 40 μM. The CCK-8 assay results showed that tBHQ prevented HG-induced cell death, with an optimal dose at 20 μM tBHQ (Figure 4A). Additionally, as indicated in Figure 4B and 4C, our immunofluorescence results showed that HG clearly decreased the protein expression of Nrf2 in podocytes, whereas tBHQ treatment significantly enhanced the expression of Nrf2 and promoted Nrf2 nuclear translocation in HG-treated podocytes. In parallel, twestern blot analysis also confirmed that tBHQ significantly increased the protein expression of nuclear Nrf2 in podocytes under HG stimulation (Figure 4D and 4F). Moreover, we found that tBHQ treatment markedly increased the expressions of Nrf2 and HO-1 and significantly decreased the expressions of NOX2 and NOX4 in HG-induced podocytes, as reflected by western blotting (Figure 4E). Furthermore, we found that tBHQ treatment significantly inhibited NADPH oxidase activity and attenuated intracellular ROS production in HG-treated podocytes (Figure 4K and 4L).

3.5. Activation of the Nrf2 signalling pathway with tBHQ attenuated HG-induced podocyte injury

Next, we wanted to further investigate the effect of tBHQ on cell injury in HG-treated podocytes. First, we found that tBHQ significantly increased the expression of podocin (Figure 5A) and attenuated cytoskeletal damage (Figure 5C) in podocytes under HG stimulation. In addition, we also evaluated the levels of apoptosis-associated proteins. The Bcl-2 family of proteins consists of proapoptotic members such as Bax as well as antiapoptotic members such as Bcl-2, which play a crucial role in regulating cell apoptosis [20]. We found that HG significantly increased the expressions of Bax and cleaved-caspase3 and decreased the expression of Bcl-2, whereas tBHQ markedly decreased the expressions of Bax and cleaved-caspase3 and increased the expression of Bcl-2 in HG-treated podocytes (Figure 5D). Moreover, flow cytometry analysis showed that tBHQ significantly attenuated HG-induced podocyte apoptosis (Figure 5H).

3.6. Effect of tBHQ on renal function and histopathological changes in STZ-induced diabetic rats

Next, we examined whether activation of the Nrf2/HO-1 signalling pathway with tBHQ ameliorated renal damage in STZ-induced DN rats. No significant difference was found in body weight between the DN rats

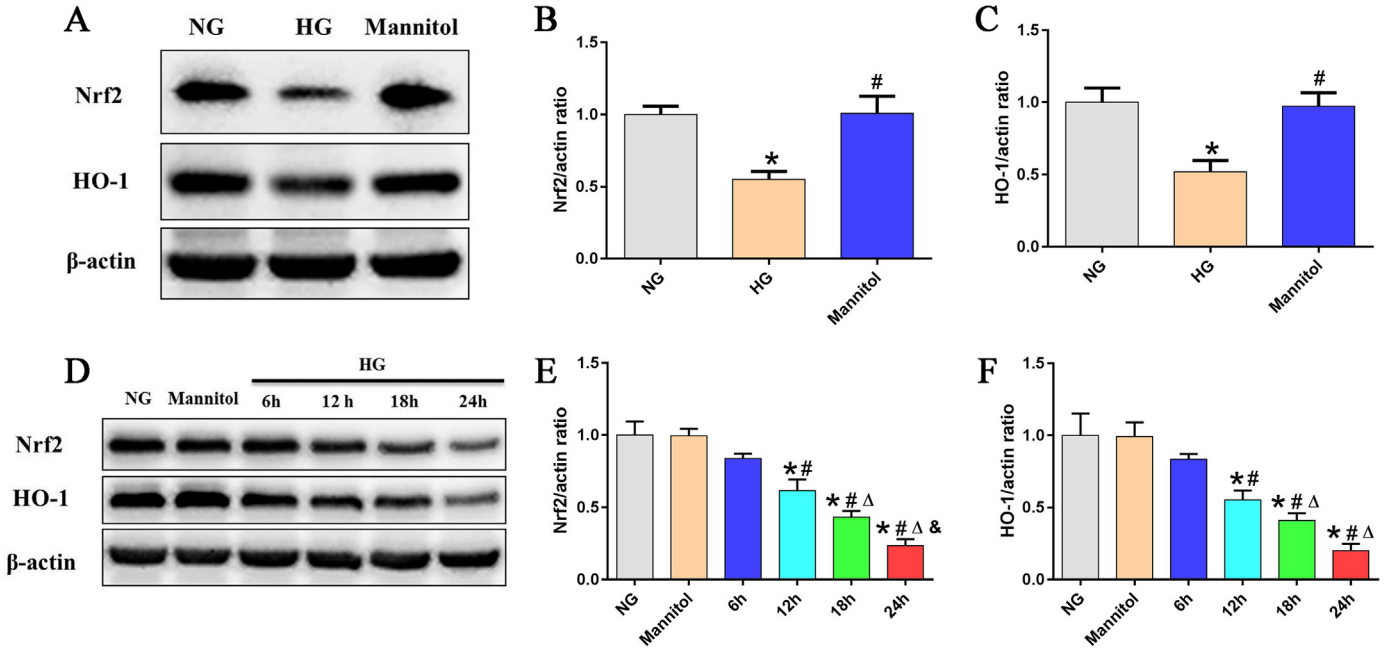

Figure 3. HG conditions inhibited the Nrf2/HO-1 signalling pathway in podocytes. (A) Western blot analysis of Nrf2 and HO-1 expression in podocytes after treatment with HG or mannitol for 24 h. (B–C) Quantification of Nrf2 and HO-1 expression. *P < 0.05 vs. NG group, $^{\#}$P < 0.05 vs. HG group. (D) Western blot analysis of Nrf2 and HO-1 expression in podocytes after treatment with HG for different time points. (E–F) Quantification of Nrf2 and HO-1 expression. *P < 0.05 vs. NG group, $^{\#}$P < 0.05 vs. mannitol group, $^{\triangle}$P < 0.05 vs. 6 h group, and $^{\&}$P < 0.05 vs. 12 h group. The uncropped images of (A and D) were referred to in supplementary Figure 2.

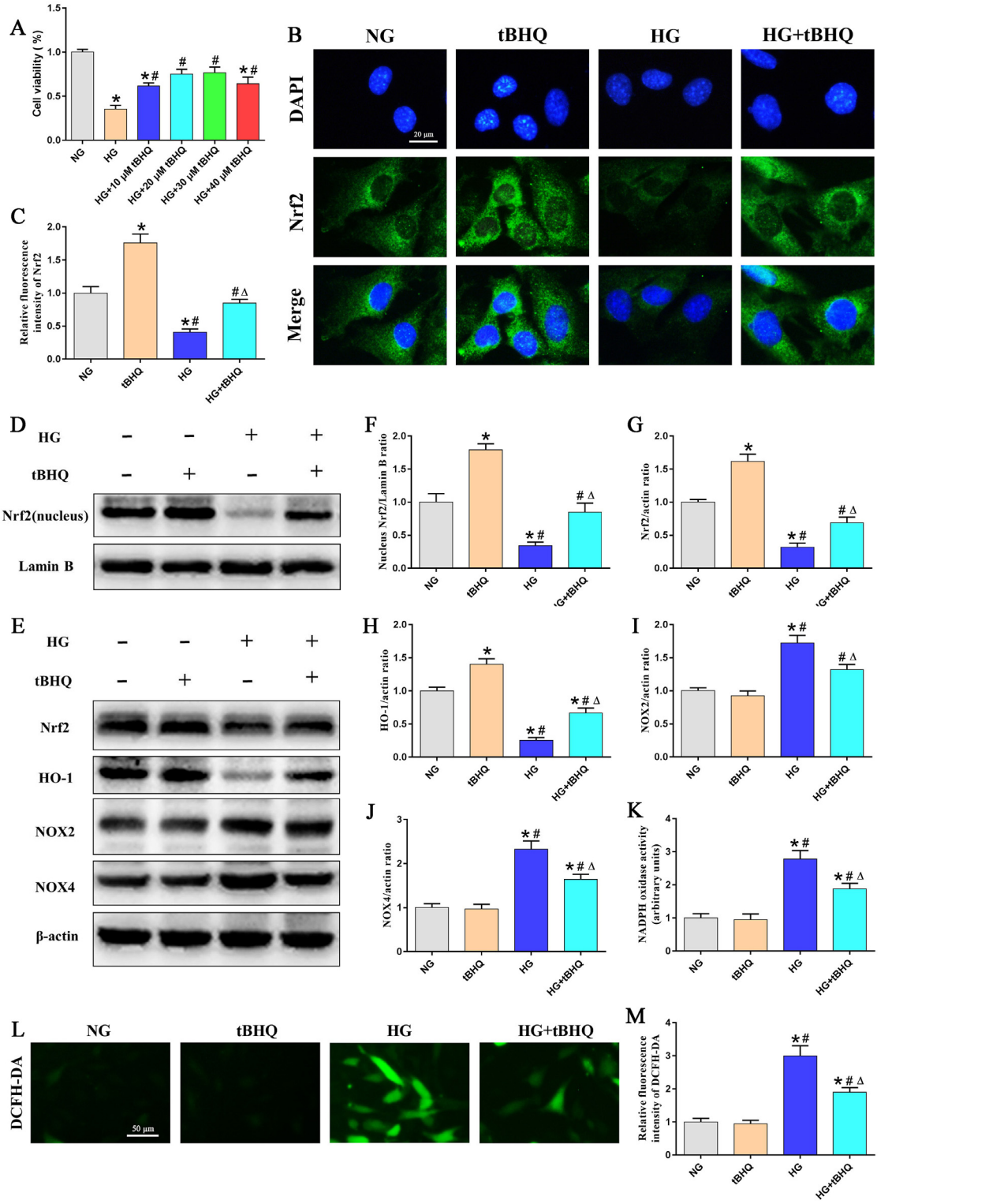

Figure 4. Activation of the Nrf2 signalling pathway with tBHQ inhibited NADPH oxidase activity and attenuated ROS production in HG-treated podocytes. (A) HK-2 cells were pretreated with different concentrations of tBHQ (0, 10, 20, 30 and 40 μM) for 2 h and then treated with HG for 24 h. Cell viability was assessed using a CCK-8 assay. (*P < 0.05 vs. NG group, $^{\#}$P < 0.05 vs. HG group). (B) Podocytes were pretreated with tBHQ (20 μM) for 2 h and then treated with or without HG for 24 h. The cells were incubated with Nrf2 antibody and DAPI. (C) Quantitative analysis of the relative fluorescence intensity of Nrf2. (D) Western blot analysis of nuclear Nrf2 expression in podocytes treated as indicated above. (E) Western blot analysis of Nrf2, HO-1, NOX2 and NOX4 expression in podocytes treated as indicated above. (F) Quantification of nuclear Nrf2 expression. (G–J) Quantification analysis of nuclear Nrf2, HO-1, NOX2 and NOX4 expression from Panel E. (K) Podocytes were treated as indicated above, and NADPH oxidase activity was determined using an NADPH oxidase activity assay kit. (L) Podocytes were treated as indicated above, and intracellular ROS production was measured with DCFH-DA staining. (M) Quantitative analysis of the relative fluorescence intensity of DCFH-DA. (*P < 0.05 vs. NG group, $^{\#}$P < 0.05 vs. tBHQ group, and $^{\triangle}$P < 0.05 vs. HG group.) The uncropped images of (D and E) were referred to in supplementary Figure 3.

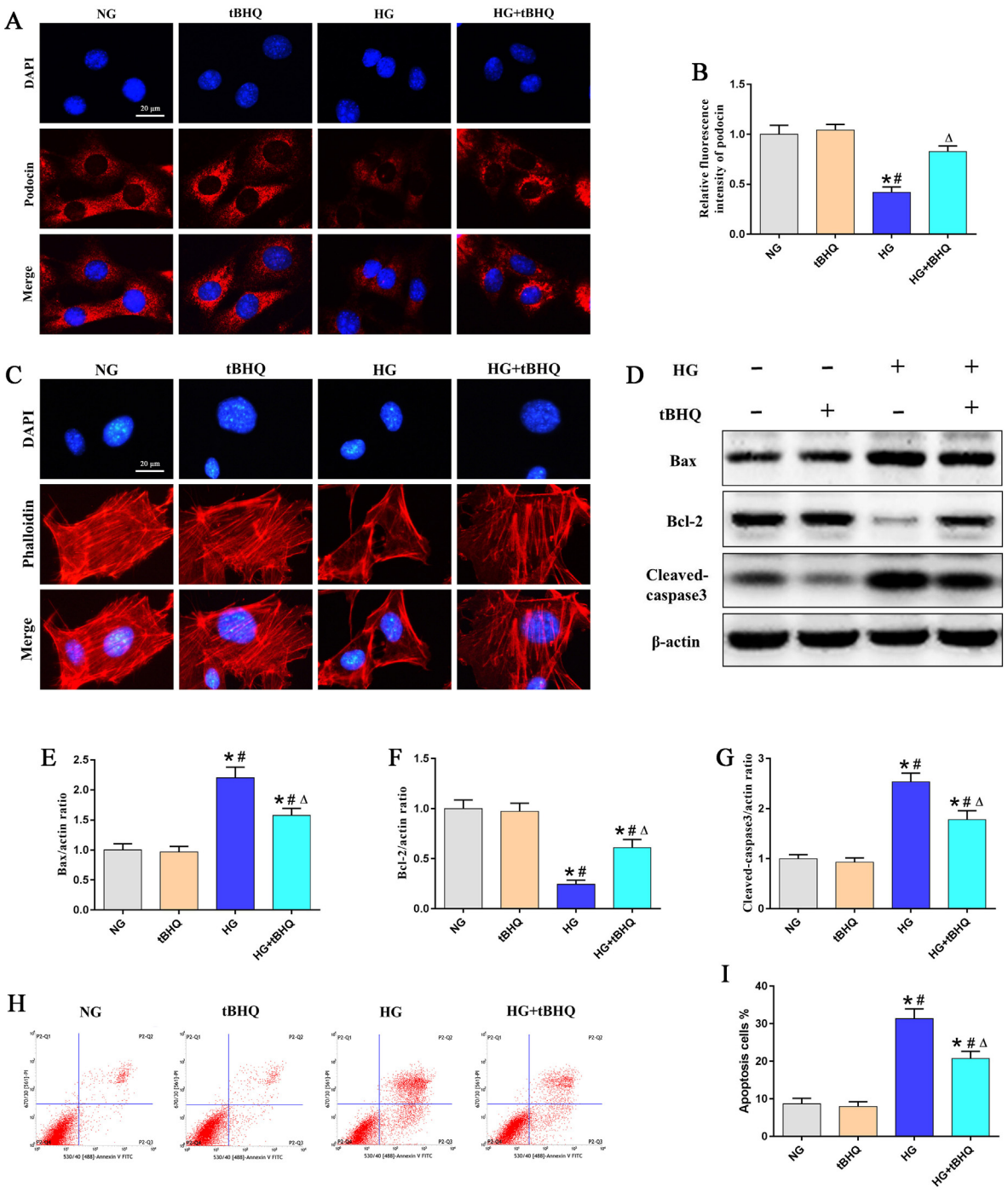

Figure 5. Activation of the Nrf2 signalling pathway with tBHQ attenuated HG-induced podocyte injury. (A) Podocytes were pretreated with tBHQ (20 μM) for 2 h and then treated with or without HG for 24 h. The cells were incubated with podocin antibody and DAPI. (B) Quantitative analysis of the relative fluorescence intensity of podocin. (C) Podocytes were treated as indicated above and stained with phalloidin. (D) Western blot analysis of Bax, Bcl-2 and cleaved caspase-3 in podocytes of different groups. (E–G) Quantification analysis of Bax, Bcl-2 and cleaved caspase-3 expression. (H) Podocytes were treated as indicated above, and flow cytometry analysis of podocyte apoptosis was performed in different groups. (I) Quantitative analysis of apoptotic cells in Figure H. (*P < 0.05 vs. NG group, $^{\#}$P < 0.05 vs. tBHQ group, and $^{\triangle}$P < 0.05 vs. HG group.) The uncropped images of (D) were referred to in supplementary Figure 4.

treated with tBHQ and the untreated DN rats (Figure 6A). Compared with the control groups, the blood glucose levels (Figure 6B), serum creatinine (SCr) (Figure 6C), blood urea nitrogen (BUN) (Figure 6D) and 24 h urinary protein (Figure 6E) were all significantly increased in the DN groups. However, tBHQ treatment significantly attenuated these renal functional parameters in DN rats. Moreover, the DN rats had a higher ratio of kidney/body weight (Figure 6F and 6G) than the control rats; however, tBHQ treatment partially reversed this trend in DN rats. Furthermore, H & E staining showed that DN rats exhibited an increase in glomerular mesangial matrix proliferation and cytosolic vacuolar formation in the proximal tubules (Figure 6H). Simultaneously, TEM examination revealed evident effacement of foot processes in DN rats (Figure 6I). However, these pathological changes in DN rats were partially attenuated by treatment with tBHQ. Taken together, these results demonstrate the beneficial effect of tBHQ on renal damage in STZ-induced DN rats.

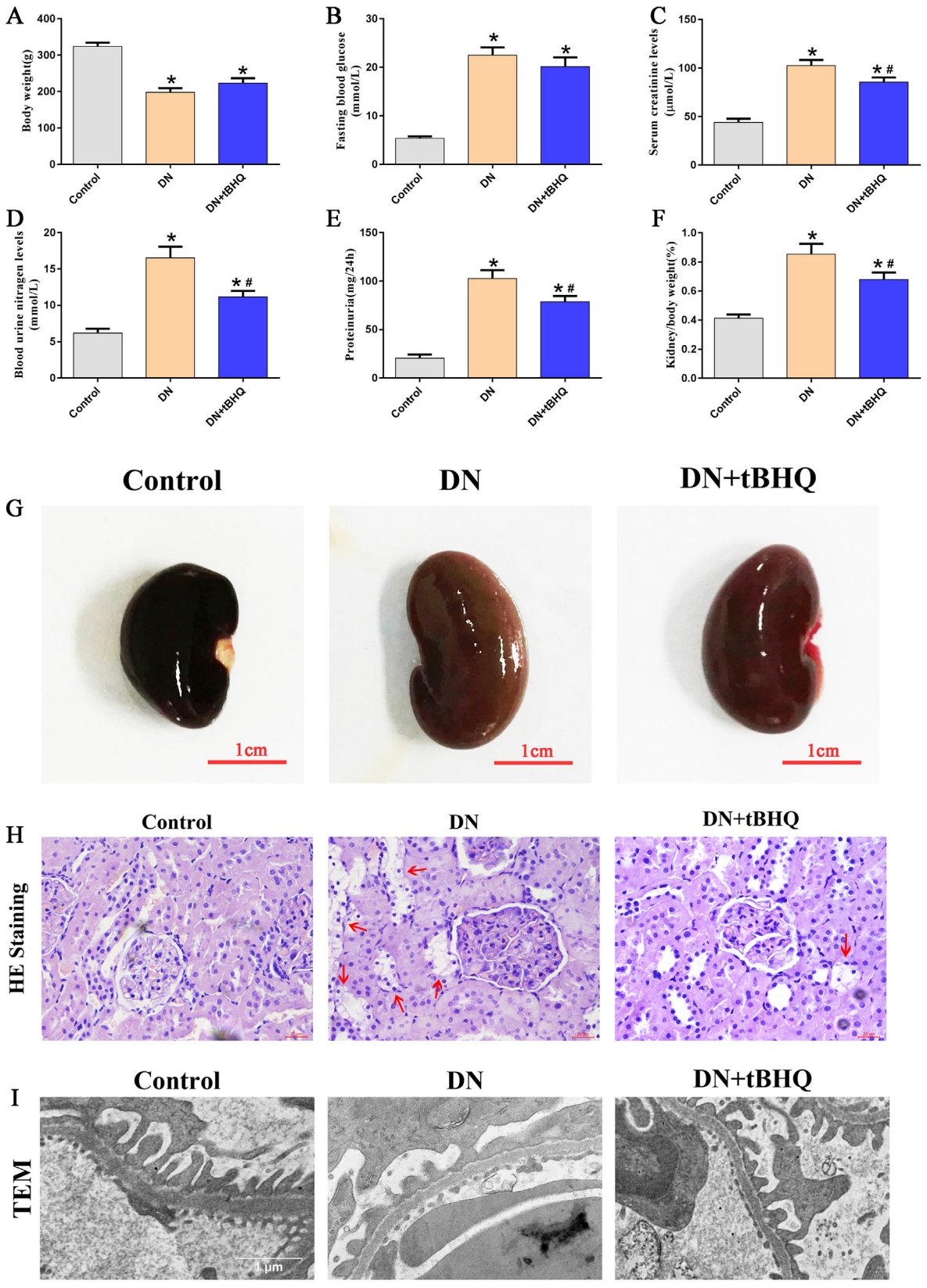

Figure 6. Effect of tBHQ on renal function and histopathological changes in STZ-induced diabetic rats. (A) Body weight. (B) Fasting blood glucose level. (C) SCr levels. (D) BUN levels. (E) Twenty-four-hour urine protein levels. (F) Kidney/body weight (%). (G) Kidney volume. (H) Representative images of HE–stained kidney sections from different groups. The red arrow indicates cytosolic vacuolar formation in the proximal tubules. (I) Representative TEM images from different groups. (*$P < 0.05$ vs. Control group, and $^{\#}P < 0.05$ vs. DN group.)

3.7. Activation of the Nrf2 signalling pathway with tBHQ inhibited renal NADPH oxidase activity and attenuated ROS generation in STZ-induced diabetic rats

Western blot analyses showed that the expressions of Nrf2 and HO-1 were significantly decreased and the expressions of NOX2 and NOX4 in the kidneys of DN rats were significantly increased in comparison with the control group (Figure 7A). However, tBHQ treatment significantly increased the expressions of Nrf2 and HO-1 and decreased the expressions of NOX2 and NOX4 in the kidneys of DN rats. Furthermore, tBHQ treatment significantly decreased the expressions of Bax and cleaved caspase-3 and increased the expression of Bcl-2 in the kidneys of DN rats (Figure 7A). In addition, immunohistochemistry staining also confirmed that tBHQ treatment markedly increased the expression of Nrf2 and decreased the expressions of NOX2 and NOX4 in the kidneys of DN rats (Figure 7C). Subsequently, we measured the level of oxidative stress in

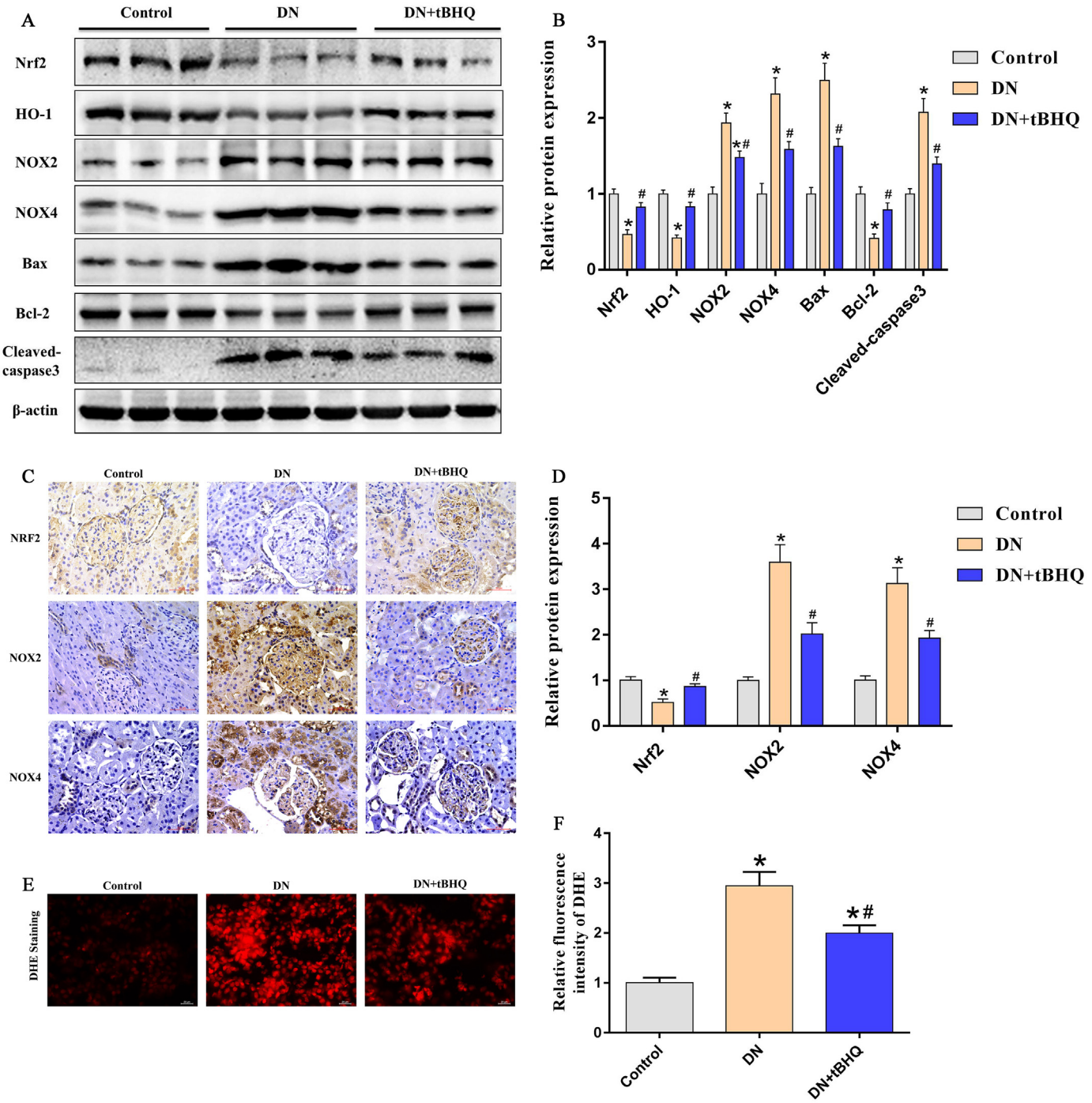

Figure 7. Activation of the Nrf2 signalling pathway with tBHQ inhibited renal NADPH oxidase activity and attenuated ROS generation in STZ-induced diabetic rats. (A) Western blot analysis of Nrf2, HO-1, NOX2, NOX4, Bax, Bcl-2 and cleaved-caspase3 expression in kidney tissues from different groups. (B) Quantification analysis of protein expression from Panel A. (C) Representative immunohistochemical staining for Nrf2, NOX2 and NOX4 in kidney tissues from different groups. (D) Quantification analysis of Nrf2, NOX2 and NOX4 expression in Panel C. (E) Representative images of DHE staining from different groups. (F) Quantitative analysis of the relative fluorescence intensity of DHE. (*P < 0.05 vs. Control group, #P < 0.05 vs. DN group.) The uncropped images of (A) were referred to in supplementary Figure 5.

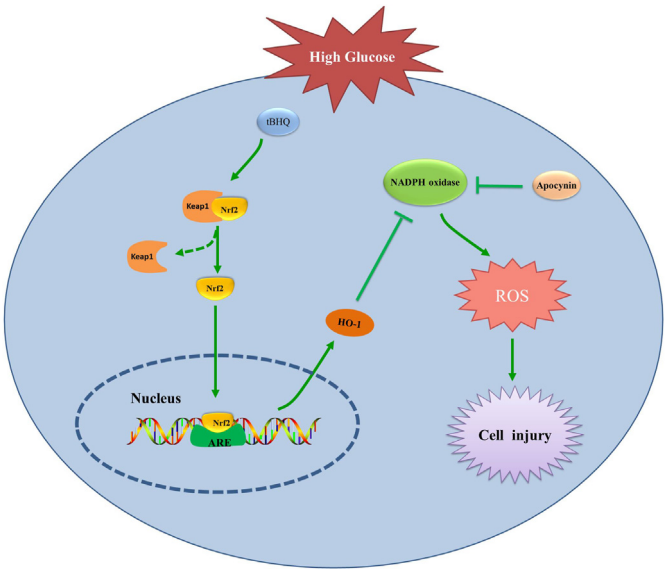

Figure 8. The hypothetical mechanisms illustrating that activation of the Nrf2/HO-1 signalling pathway with tBHQ attenuates podocyte injury in diabetic nephropathy by inhibiting NADPH oxidase-derived ROS generation. HG exposure inhibits the Nrf2/HO-1 antioxidant signalling pathway and enhances NADPH oxidase activity, which eventually leads to ROS overproduction and cell apoptosis. However, tBHQ upregulates the Nrf2/HO-1 antioxidant signalling pathway, which subsequently inhibits NADPH oxidase-derived ROS generation, thereby alleviating hyperglycaemia-induced podocyte apoptosis.

kidney tissue via DHE staining and found that renal oxidative stress levels were significantly enhanced in the kidneys of DN rats compared with those of control rats (Figure 7E); however, this enhancement was dramatically alleviated following tBHQ administration.

4. Discussion

DN is a devastating complication of diabetes mellitus and is associated with increased mortality in diabetic patients [21]. Podocyte dysfunction has been identified as an important contributor to the pathogenesis and progression of DN [22]. Therefore, protecting podocytes from damage is an effective strategy to improve kidney function in DN.

NADPH oxidase is a major source of ROS generation, and it plays an essential role in mediating oxidative stress-induced cell injury and death [23]. Mounting evidence indicates that NADPH oxidases play a critical role in the progression of DN, and inhibition of NADPH oxidase activity alleviates kidney injury in DN [24, 25]. Previous studies have shown that HG increases NADPH oxidase activity [26] and NOX4 expression [27] in podocytes, and podocyte-specific Nox4 deletion reduces ROS production and attenuates podocyte injury and albuminuria in streptozotocin-induced diabetic mice [28]. Cui et al. found that pharmacologic inhibition of NOX4 expression attenuated HG-induced podocyte apoptosis by inhibiting the ROS/p38 signalling pathway [29]. Consistent with those studies, our present results proved that HG enhanced NADPH oxidase activity, increased the expressions of NOX2 and NOX4, and promoted ROS production in podocytes both in vitro and in vivo. Moreover, we also found that inhibition of NADPH oxidase activity with apocynin significantly alleviated HG-induced ROS generation, cytoskeletal damage and cell apoptosis in podocytes, indicating that NADPH oxidase-derived ROS mediated HG-induced podocyte injury. Thus, regulating NADPH oxidase activity and reducing excessive ROS generation may be a potential strategy to protect HG-induced podocyte injury and delay the progression of diabetic nephropathy.

The Nrf2 signalling pathway can efficiently counteract the generation of ROS and plays a crucial role in maintaining cellular redox homeostasis [30]. Previous studies have demonstrated that knocking out Nrf2 enhances renal oxidative stress and accelerates renal injury in STZ-induced diabetic mice [31], whereas activation of the Nrf2 signalling pathway ameliorates the development of STZ-induced DN in rats [32], suggesting a renoprotective effect of the Nrf2 pathway in DN. In our study, we found that the Nrf2/HO-1 antioxidant pathway was significantly suppressed in HG-treated podocytes and in the kidneys of STZ-induced diabetic rats, as shown by a reduction in the expressions of Nrf2 and HO-1. Our results are consistent with these previous studies, which showed that the Nrf2 signalling pathway is inhibited in animal models of DN [32, 33, 34]. However, it is interesting to note that upregulation of the Nrf2 signalling pathway is also observed in animal models of DN [35]. The significant variation in the literature may be attributed to differences in the experimental animals, methods and observation periods, the effect of which need to be further elucidated in future studies. The imbalance caused by excessive ROS generation and an inadequate endogenous Nrf2/HO-1 antioxidant pathway will inevitably result in intracellular oxidative stress and induce cell injury and podocyte death under hyperglycaemic conditions. Therefore, promoting the activation of the Nrf2/HO-1 signalling pathway might represent a potential therapeutic strategy to protect against HG-induced podocyte injury.

The compound tBHQ is known to be an inducer of Nrf2, and numerous previous studies have reported that tBHQ exerts remarkable antioxidant activity in various kidney diseases, including cisplatin-induced nephrotoxicity [36], ischaemia–reperfusion-induced kidney injury [37] and DN [38]. In the present study, we found that tBHQ treatment significantly activated the Nrf2 signalling pathway as evidenced by increased expressions of Nrf2 and HO-1 in the kidneys of diabetic rats and in HG-induced podocytes. Our results showed that tBHQ treatment enhanced Nrf2 nuclear translocation in HG-treated podocytes, indicating the upregulation of the Nrf2 signalling pathway. Moreover, we found that tBHQ partially preserved renal function in diabetic rats and alleviated HG-induced cytoskeletal damage and cell apoptosis in podocytes, suggesting that tBHQ has significant beneficial effects on podocyte survival and ameliorates the development of DN. However, little is known about the exact regulatory molecular mechanisms of tBHQ in podocytes under hyperglycaemic conditions. Several previous studies have focused on the possible relationships between the Nrf2 signalling pathway and NADPH oxidase. Dai et al. reported that activation of the Nrf2 signalling pathway with isoquercetin attenuated oxidative stress and neuronal apoptosis via inhibition of the NOX4/ROS/NF-κB signalling pathway in a rat model of cerebral I/R injury [39]. Wei et al. showed that Nrf2 deficiency delayed revascularization by upregulating the expression of NOX2 and enhancing oxidative stress in a mouse model of oxygen-induced retinopathy [40]. In the present study, we found that activation of the Nrf2 signalling pathway with tBHQ significantly inhibited NADPH oxidase activity and decreased the expressions of NOX2 and NOX4 in the kidneys of diabetic rats and in HG-induced podocytes. Furthermore, we also found that activation of the Nrf2 signalling pathway with tBHQ significantly attenuated ROS generation both in vitro and in vivo. Thus, the exact protective mechanisms of tBHQ in HG-induced podocyte injury may involve the inhibition of NADPH oxidase activity by activating the Nrf2 signalling pathway, resulting in a subsequent decrease in ROS production and thereby promoting the survival of podocytes.

5. Conclusions

In summary, the present study demonstrated that HG increased NADPH oxidase activity and ROS production, inhibited the Nrf2/HO-1 antioxidant pathway, and induced cytoskeletal damage and apoptosis in podocytes. Activation of the Nrf2/HO-1 signalling pathway with tBHQ attenuated HG-induced podocyte injury via inhibition of NADPH oxidase-derived ROS production (Figure 8). Our results provide new insights into the molecular mechanisms underlying the renoprotective effects of tBHQ in DN and highlight the potential of upregulation of the Nrf2/HO-1 antioxidant pathway with tBHQ as a therapeutic approach in DN.

Declarations

Author contribution statement

Ting Liu: Performed the experiments; Wrote the paper. Chang-yan Li; Juan Liu: Contributed reagents, materials, analysis tools or data. Li-li Zhong, Ming-min Tang; Hao Chen; Wen-bo Wang: Analyzed and interpreted the data. Jin-ping Huang: Conceived and designed the experiments. Xu-shun Jiang: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

Funding statement

Chang-yan Li was supported by Health and Family Planning Commission of Sichuan Province [No. 2019094].

Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2022.e10515.

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