

Original Paper

Crocin Protects Podocytes Against Oxidative Stress and Inflammation Induced by High Glucose Through Inhibition of NF- κ B

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Background/Aims: Diabetic nephropathy (DN) is a microangiopathic disease characterized by excessive urinary albumin excretion, which occurs in 30% of patients with diabetes mellitus. It is the second leading cause of end-stage renal diseases in China. Nuclear factor-kappa B (NF- κ B) is reported to be closely correlated with the inflammation underlying diabetes-associated renal damage. Crocin, a plant-derived compound, has antioxidant properties that may inhibit NF- κ B. **Methods:** In the present study, we used a conditionally immortalized mouse podocyte cell line to explore whether crocin could effectively block albuminuria. Cells were incubated with 15 or 25 mM D-glucose to mimic diabetic conditions. The expression of Wilms tumor 1 (WT-1) and synaptopodin was evaluated to identify differentiated podocytes, and the expression of nephrin, podocin, and CD2ap was measured as markers of slit diaphragms, the main structures within the glomerular filtration barrier. **Results:** The high-glucose conditions led to reduced nephrin, podocin, and CD2ap expression, which was prevented by pretreatment with crocin. The oxidative stress and pro-inflammatory response of podocytes associated with DN induced by high glucose were also reduced by crocin pretreatment. Phosphorylated I κ B α (p-I κ B α) expression induced by high glucose was also significantly decreased by crocin pretreatment. Moreover, pyrrolidine dithiocarbamate, a NF- κ B inhibitor, pyrrolidine dithiocarbamate, augmented the protective effects of crocin. **Conclusion:** Our results demonstrate a protective role of crocin against damage to podocytes and slit diaphragms under high-glucose conditions via inhibition of NF- κ B. This study presents a potential therapy for DN and contributes to the understanding of the mechanism underlying DN.

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Introduction

Diabetes mellitus (DM), a disease with complex mechanisms, is characterized by variable and aberrant glucose metabolism. Multiple complications are associated with DM, the most prevalent of which is nephropathy, with a morbidity of 25–40% [1, 2]. Diabetic nephropathy (DN), which is characterized by glomerular and tubular disorders, is a major microangiopathic complication of DM that occurs in up to 30% of patients [3]. Furthermore, DN is also a major cause of end-stage renal diseases (ESRD) in China, ranking second only to glomerulonephritis [4]. Once early DN has developed into ESRD, kidney failure is unavoidable and irreversible, requiring renal replacement therapy [5, 6]. Hence, early diagnosis, intervention, and treatment are critical for reversing or halting the progression of DN. Elevated proteinuria secretion ($20 \mu\text{g}/\text{min} < \text{urine albumin excretion rate} < 200 \mu\text{g}/\text{min}$) due to decreased glomerular filtration rate is the major clinical and recognized hallmark of early DN [7]. It results from damage to the filtration barrier by chronic hyperglycemia and the subsequent alteration of renal hemodynamics. The American Diabetes Association recommends strict glycemic control as the first-line method for the prevention of diabetic complications [5]; however, this approach does not completely prevent the progression of DN [8]. Moreover, while some therapeutic methods can relieve DN symptoms, including strict restriction of blood pressure, administration of angiotensin-converting enzyme inhibitors, and pharmacological suppression of the renin-angiotensin system, none of the available drugs are able to efficiently prevent or cure DN. Aside from the inadequacy of current drug therapies, knowledge of mechanisms underlying the progression of DN is also insufficient. Hence, investigation into the progression of DN and development of new drugs is urgently needed.

Podocytes (glomerular epithelial cells) are highly specialized and terminally differentiated cells that play an important role in the formation of the glomerular filtration barrier [9]; therefore, they are critical in protecting against proteinuria. The foot processes of podocytes are interlinked by slit diaphragms (SD), which are essential structures of the selective filtration barrier in glomeruli [10–12]. Changes in SD are generally associated with proteinuric diseases [11, 13]. Because mature podocytes do not have a proliferative capacity, damage to these cells may induce continuous and irreversible albuminuria [11, 12]. Studies have shown that podocytes are injured during the development of DN [14], hence, reducing podocyte injury has been proposed as an effective approach to prevent or slow the progression of DN.

Saffron (*Crocus sativus* L.) is a traditional Chinese medicine frequently used in clinical therapy for treatment of diseases [15], including, but not limited to, tumors [16], hypertension [17], insomnia and anxiety [18], cerebral ischemia [19], and depression [20]. Saffron extracts have been shown to have beneficial effects, including anti-tumor, anti-oxidation, anti-senescence, and pro-cardiovascular effects [21, 22]. As previously reported, crocin is one of the two most pharmacologically bioactive constituents found in gardenia fruits and saffron stigmas [23] with antioxidant properties, anti-apoptotic actions, and anti-inflammatory effects being attributed to it [24, 25]. However, whether crocin has a beneficial effect on podocytes remains unknown.

Numerous studies have shown that oxidative stress is a major contributor to the pathogenesis of DN [26, 27]. The kidney remodeling and tissue fibrosis induced by hyperglycemia-induced oxidative damage contribute greatly to the development and progression of DN [28]. Miranda-Diaz et al. reported an increase in reactive oxygen species (ROS) in diabetic mice with nephropathy [29], and Siddiqi et al. demonstrated oxidative injury in podocytes cultured under high-glucose conditions [30]. In addition, altered secretion of pro-inflammatory factors, including interleukin (IL)-8 and IL-10, is observed during the early stages of DN, and these factors are major contributors to the pathogenesis of DN [31]. Inhibition of podocyte inflammation induced by fructose [32] or lipopolysaccharides [33] appears to be a promising therapeutic strategy for the prevention of podocyte injury [34]. Whether crocin plays a similar role in relieving the oxidative stress and pro-inflammatory

response of podocytes during the progression of DN is unclear. Nuclear factor-kappa B (NF-κB) is a ubiquitous and important nuclear transcription factor that regulates the expression of a large number of genes involved in inflammation [35] and the inflammatory response [36]. Hence, it is also of great importance to explore the relationship between NF-κB and inflammation in podocytes, the understanding of which is limited.

In summary, it is well-known that podocyte injury disrupts the filtration barrier and contributes to albuminuria in patients with DN, but the impact of crocin and NF-κB on this process remains unknown. This study aimed to investigate whether crocin has a protective effect on podocytes cultured under hyperglycemic conditions, and if so, to elucidate the underlying mechanism and identify a potential therapeutic strategy for the treatment of DN.

Materials and Methods

Culture of mouse podocytes

Mouse podocytes were cultured as previously described [3, 37]. Briefly, to induce proliferation, culture dishes were precoated and incubated with type 1 collagen (Sigma, St. Louis, MO, USA) overnight, and cells were cultured in type 1 collagen-coated plates containing RPMI-1640 medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone) and 10 U/ml interferon (IFN)-γ (Sigma) at 33 °C with 5% CO₂. To induce differentiation, podocytes were cultured at 37 °C with 5% CO₂, without IFN-γ, for 14 days. Differentiated podocytes were serum starved for 24 h in RPMI-1640 medium containing 0.5% FBS prior to the experiments described below.

Cell treatment

To mimic *in vivo* diabetic conditions, differentiated podocytes were seeded in type 1 collagen-coated plates and cultured in medium supplemented with either 15 or 25 mM D-glucose (Sigma) for 7 days. Cells cultured in a normal glucose concentration (5 mM D-glucose) served as the control. For certain groups, podocytes were pretreated with 0.1, 0.5, or 1 μM of crocin (Sigma) for 24 h prior to exposure to high glucose. Some subsamples within the crocin-treated groups were pretreated with NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC; 10 μM; Sigma) for 2 h prior to incubation with crocin (0.1, 0.5, or 1 μM) for 24 h, followed by incubation with D-glucose (15 or 25 mM).

MTT assay

The MTT assay was performed using a MTT Cell Proliferation and Cytotoxicity Assay Kit (Beyotime, Shanghai, China). Briefly, cells were seeded onto 96-well plates, followed by incubation with MTT solution for 48 h, to which a dissolving solution was added to dissolve the formazan formed. The optical density (OD) value recorded at 490 nm was used to monitor cell viability.

Reactive oxygen species assay

The ROS assay was performed using a Reactive Oxygen Species Assay Kit (Beyotime). Briefly, cells were digested and harvested, then stained with dichloro-dihydro-fluorescein diacetate (DCFH-DA) according to the manufacturer's instructions. The signal was detected with an excitation wavelength of 488 nm and an emission wavelength of 525 nm by using flow cytometer (FACS Calibur, BD Biosciences, San Jose, CA, USA). Flow cytometry data were analyzed using CellQuest Pro software (BD Biosciences). Cells treated with ROSup only (provided with the kit) were used as the positive control.

Superoxide dismutase assay

The superoxide dismutase (SOD) assay was performed using a Total Superoxide Dismutase Assay Kit (Beyotime). Briefly, the supernatant obtained from cell homogenates was mixed with the working solution, and the mixtures were incubated at 37 °C for 30 min. The OD value was measured at 450 nm. The SOD level was calculated according to the manufacturer's instructions.

ELISA

The protein levels of IL-1β, IL-8, IL-10, and tumor necrosis factor (TNF)-α in cell supernatant were measured using ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's

instructions. Briefly, cells were digested and centrifuged, and the culture supernatant was collected to determine the protein concentrations using a SmartSpec Plus spectrophotometer (Bio-Rad, Hercules, CA, USA).

Quantitative PCR

Total RNA was extracted using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed into cDNA using SuperScript® VILO cDNA Synthesis Kit and Master Mix (Invitrogen). Quantitative PCR was performed using SYBR® Fast qPCR Mix (Takara Biotechnology, Dalian, China) and a CFX96 Real-Time PCR System (Bio-Rad). All procedures were performed following the manufacturer's instructions. The relative expression compared to the reference gene β-actin was calculated using the $2^{-\Delta\Delta Ct}$ method. The primer sequences are shown in Table 1.

Western blotting

Proteins were extracted using RIPA buffer (Sigma), then subjected to 10% SDS-PAGE, followed by transfer onto nitrocellulose (NC) membranes (Merck Millipore, Billerica, MA, USA). After being blocked with 10% skim milk in TBS-T, the membrane was incubated with the primary antibodies in Table 2 overnight at 4 °C. The membrane was then incubated with horseradish peroxidase-conjugated secondary antibodies (catalog No. G-21234, 1:2000, Invitrogen) for 2 h at room temperature. Bands were detected using an enhanced chemiluminescence kit (Invitrogen). The OD analysis was performed using Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA).

Statistical analysis

Data are displayed as mean ± standard deviation (SD). Comparison between two groups was performed using Student's *t*-test, and analysis of variance (ANOVA) was used for multiple groups. For all analyses, $p < 0.05$ and $p < 0.01$ indicated statistical significance. GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA) and Adobe Photoshop CS2 software (Adobe Systems Incorporated, San Jose, CA, USA) were used to create Figures.

Results

Identification of differentiated podocytes

Podocytes were cultured at 33 °C in an undifferentiated state, then maintained at 37 °C to foster differentiation. Undifferentiated podocytes showed an epithelial-like cobblestone shape with small cell bodies (Fig. 1A). After thermoswitching to 37 °C, the cell bodies enlarged, accompanied by the formation of foot processes and spindle-like projections and junctions. As shown in Fig. 1B, differentiated podocytes could be identified by their large arborized shape and foot processes and by expression of the

Table 1. Primer sequences used in this study

Gene	Primer sequences (5'-3')	Product (bp)
WT-1	Forward: ATCCCAGGCAGGAAAGTGTG	126
	Reverse: TGCAGTCAATCAGGTGTCT	
Synaptopodin	Forward: CCGTGAGTCCCCTTACAGC	234
	Reverse: CCTCACTAAGCCCCGAGCTA	
Nephrin	Forward: TTGTAATGGGGCTTCTCCCA	397
	Reverse: AACCTGCAACCCAAAATCTCAC	
Podocin	Forward: TTGAGAATGGACAGCAGGGC	293
	Reverse: TTGATGCCTTCTCTGGTCTG	
CD2ap	Forward: ATGGCCTTGTGCTGTAGCAAT	123
	Reverse: AAGGCAAGGTAGGCTAAACG	
β-actin	Forward: TGAGCTGCGTTTACACCCT	231
	Reverse: TTTGGGGGATGTTTGCTCCA	

Table 2. Primary antibodies used in this study

Antibody	Host	Cat. No.	Dilution	Supplier
WT-1	Rabbit	PA1-20991	1:1000	Invitrogen
Synaptopodin	Rabbit	PA5-21062	1:1000	Invitrogen
Nephrin	Rabbit	PA5-20330	1:1000	Invitrogen
Podocin	Rabbit	PA5-37284	1:500	Invitrogen
CD2ap	Rabbit	PA5-28391	1:1500	Invitrogen
p-IκBα	Rabbit	PA5-38603	1:1000	Invitrogen
IκBα	Rabbit	PA5-22120	1:2000	Invitrogen
β-actin	Rabbit	PA5-16914	1:1000	Invitrogen

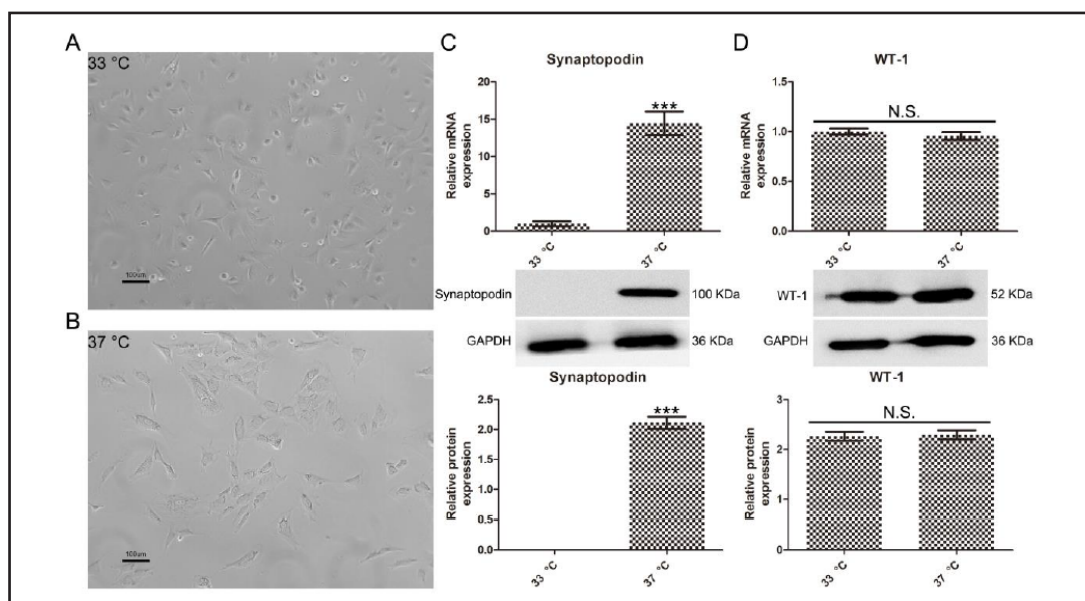
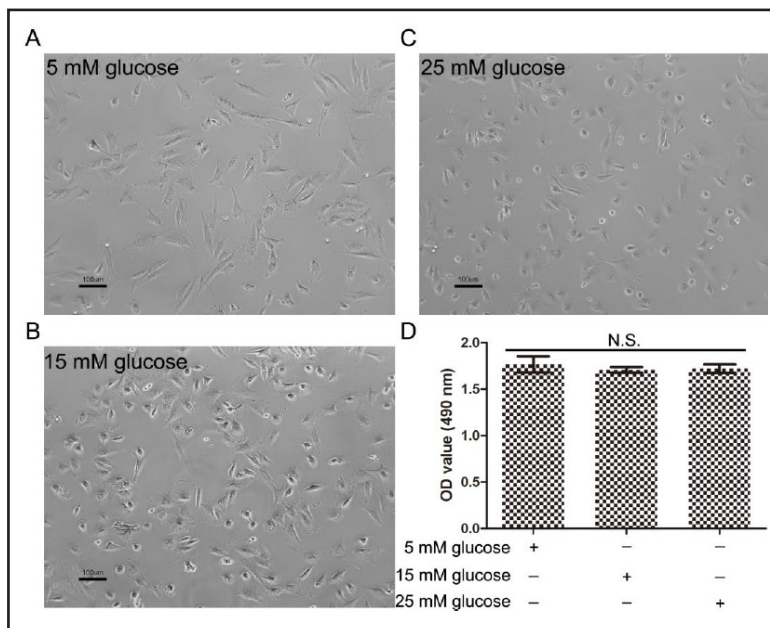


Fig. 1. Identification of differentiated podocytes. The morphology of podocytes cultured at 33 °C (A) and 37 °C (B). After thermoswitching to 37 °C, cells shifted from a cobblestone shape to an arborized shape. The mRNA and protein expression of synaptopodin (C) and WT-1 (D), as analyzed by quantitative PCR and Western blotting, respectively. Podocytes cultured at 37 °C expressed both WT-1 and synaptopodin, but there was no expression of synaptopodin at 33 °C. ***, $p < 0.01$; N.S., no statistical significance; Scale bars = 100 μ m.

Fig. 2. Construction of a diabetic model in podocytes. Cells were cultured in a medium supplemented with either 15 or 25 mM D-glucose. Cells cultured in a normal glucose concentration (5 mM D-glucose) served as the control. Morphology of podocytes cultured under 5 mM (A), 15 mM (B), and 25 mM D-glucose (C). Cells lost their intercellular junctions, with shorter foot processes and a smaller cell size. (D) The viability of podocytes was assessed by MTT assay. N.S., no statistical significance; Scale bars = 100 μ m.



differentiation marker synaptopodin [38, 39] (Fig. 1C), which is not detected in undifferentiated cobblestone-shaped podocytes [40]. Both cobblestone and arborized cells originated from podocytes, as evidenced by expression of the podocyte-specific marker WT-1 [40, 41] (Fig. 1D).

Construction of a diabetic model in podocytes

The differentiated podocytes were cultured in medium supplemented with a high glucose concentration (15 or 25 mM D-glucose) at 37 °C to mimic diabetic conditions. As

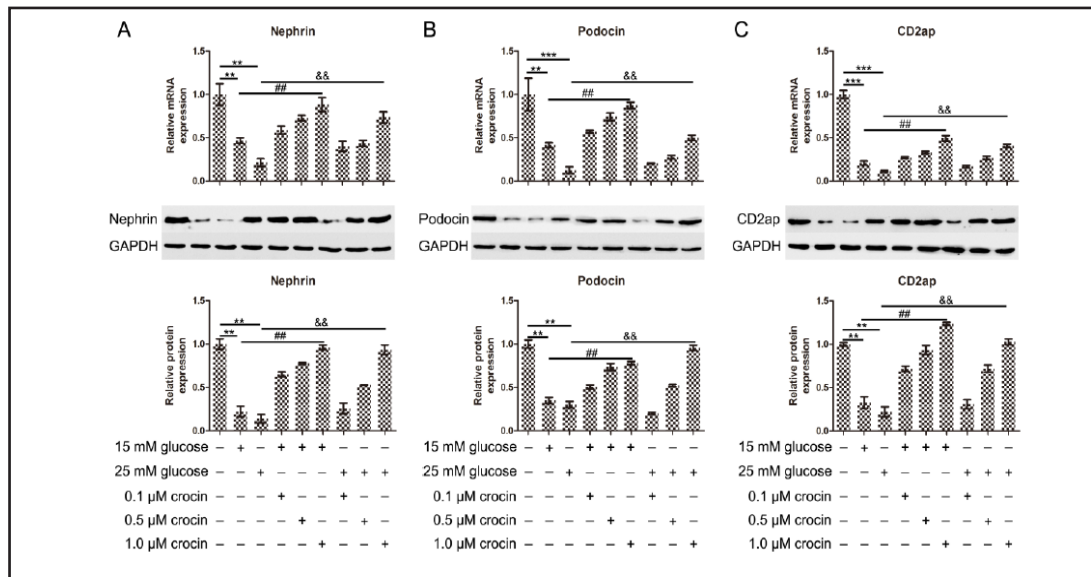


Fig. 3. Investigation of SD markers. The mRNA and protein expression of nephrin (A), podocin (B), and CD2ap (C) evaluated by quantitative PCR and Western blotting, respectively. High-glucose conditions significantly decreased the expression of these three genes, while crocin markedly restored their expression in a dose-dependent manner. ** and ## and &&, $p < 0.01$; ***, $p < 0.001$.

shown in Fig. 2A-C, cells lost their intercellular junctions after incubation with 15 or 25 mM D-glucose, with shortened foot processes and a smaller cell size. In addition, the results of the MTT assay showed that podocytes cultured under 15 or 25 mM D-glucose had similar viability compared with control cells (Fig. 2D), indicating that these cells could be used in the following experiments.

Evaluation of SD markers

Nephrin, podocin, and CD2ap are key markers for SD [13, 41]. The mRNA and protein expression of these three genes was investigated to explore the variation of SD under diabetic conditions. As shown in Fig. 3, high-glucose conditions induced a significant decrease in nephrin expression (Fig. 3A), in addition to markedly down-regulated expression of podocin (Fig. 3B) and CD2ap (Fig. 3C) at the mRNA level. Moreover, the protein expression of SD markers was also confirmed to be notably decreased, as evaluated by Western blotting and OD analysis. The effects of 0.1, 0.5, or 1 μM of crocin were explored in this study. Crocin showed a dose-dependent prevention of the inhibitory effects of high glucose on SD markers, indicating that crocin has a protective role in SD.

Evaluation of oxidative stress and pro-inflammatory response

Because we identified a protective role of crocin in SD, we explored the mechanism involved by evaluating the effect of crocin on oxidation and inflammation induced by high glucose. Unsurprisingly, 15 or 25 mM D-glucose significantly up-regulated the levels of ROS (Fig. 4A) but inhibited the production of SOD (Fig. 4B). Similarly, the levels of pro-inflammatory factors, including IL-1 β (Fig. 4C), IL-8 (Fig. 4D), IL-10 (Fig. 4E), and TNF- α (Fig. 4F), were remarkably increased in podocytes after incubation with 15 or 25 mM D-glucose.

In contrast, crocin demonstrated protective effects on podocytes by significantly inhibiting ROS production and restoring SOD levels in a dose-dependent manner. In addition, crocin notably blocked the release of IL-1 β , IL-8, IL-10, and TNF- α induced by the hyperglycemic conditions, as expected, thus inhibiting the pro-inflammatory response. Morphological alterations of podocytes after treatment with crocin were also observed. Podocytes treated with crocin demonstrated more branches even cultured under 15 mM

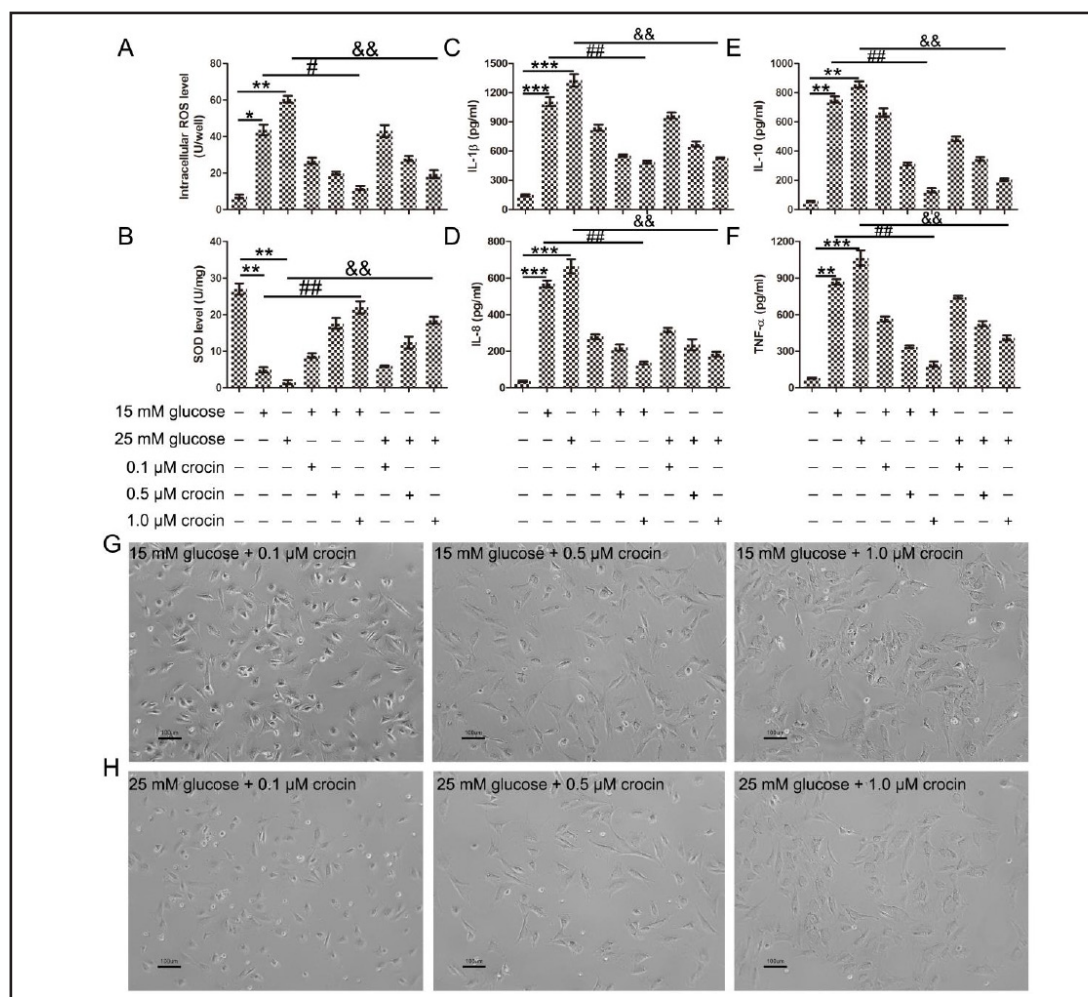


Fig. 4. Evaluation of oxidative stress and inflammatory response. Levels of reactive oxygen species (ROS; A) and superoxide dismutase (SOD; B). The pro-inflammatory factors IL-1 β (C), IL-8 (D), IL-10 (E) and TNF- α (F) were also measured by ELISA. In general, the oxidative stress and inflammation induced by high-glucose conditions were significantly ameliorated by crocin treatment in a dose-dependent manner. Podocytes cultured under 15 mM (G) or 25 mM (H) glucose went through a morphological alteration from an unbranched and smaller shape to a branched and larger shape with pretreatment with crocin. * and #, $p < 0.05$; ** and ## and &&, $p < 0.01$; ***, $p < 0.001$; Scale bars = 100 μ m.

(Fig. 4G) and 25 mM (Fig. 4H) glucose in a dose-dependent manner. In summary, crocin reversed the harmful effects of hyperglycemia on podocytes in a dose-dependent manner, thus demonstrating its positive role in reducing podocyte injury.

Evaluation of NF- κ B signaling

As mentioned previously, NF- κ B is closely related to the pro-inflammatory responses that usually trigger inflammation [36]. Hence, in this study, we studied NF- κ B signaling to investigate its degree of involvement. NF- κ B inhibitor PDTC (10 μ M) was used to treat podocytes, along with 1 μ M of crocin and 25 mM of D-glucose, the concentrations of which were selected based on above studies. As shown in Fig. 5A and B, phosphorylated I κ B α (p-I κ B α) expression was significantly up-regulated under high-glucose conditions, but markedly inhibited by pretreatment with 1 μ M of crocin. PDTC further decreased the p-I κ B α level immensely, which consequently down-regulated the levels of IL-1 β (Fig. 5C) and TNF- α

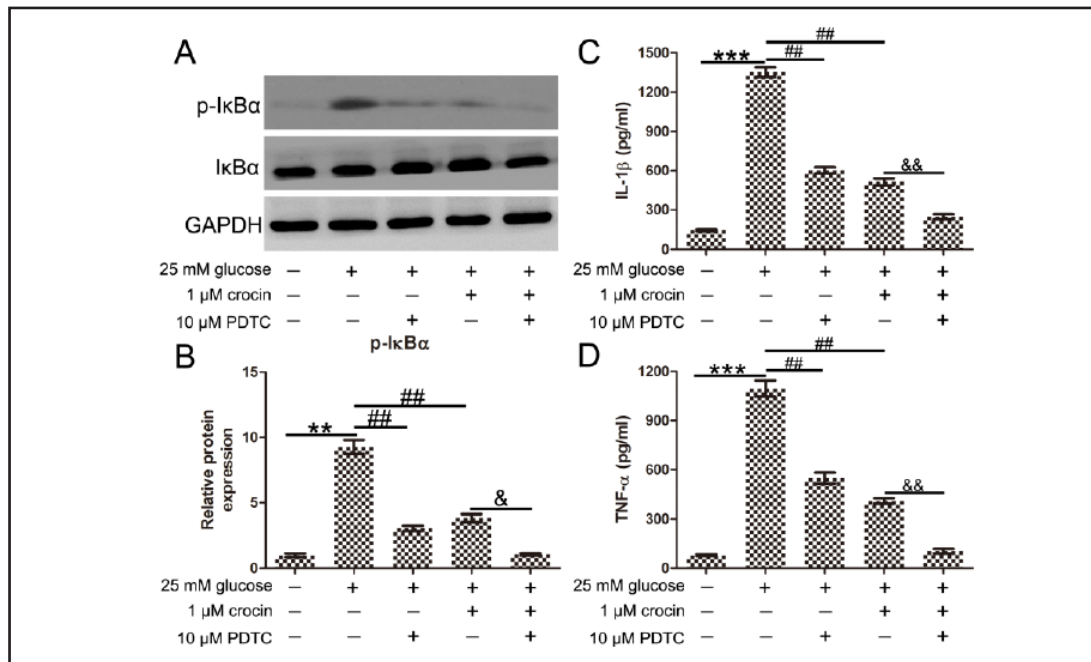


Fig. 5. Assessment of NF-κB involvement. Podocytes were treated with the NF-κB inhibitor pyrrolidine dithio carbamate (PDTC; 10 μM), crocin (1 μM) and D-glucose (25 mM). (A) Protein expression of p-IκBα and IκBα evaluated by Western blot. (B) The OD analysis of p-IκBα bands. The expression of p-IκBα was promoted by 25 mM of D-glucose, but inhibited by 1 μM of crocin and 10 μM of PDTC. The levels of IL-1β (C) and TNF-α (D) were decreased by PDTC, which reinforced the effect of crocin. *, $p < 0.05$; **, $p < 0.01$.

(Fig. 5D). These results indicate that crocin inhibits oxidative stress and the inflammatory response of podocytes through inhibition of NF-κB.

Discussion

The present study focused on an evaluation of podocytes under culture conditions that mimicked *in vivo* diabetic conditions. High levels of glucose were used to model DN in podocytes. The results showed that under hyperglycemic conditions, podocytes underwent oxidative stress and an inflammatory response, which appeared to strongly contribute to the destruction of SD (measured as decreased nephrin, podocin and CD2ap expression). Moreover, crocin demonstrated a protective role in SD, with significantly inhibited oxidative stress and decreased levels of IL-1β, IL-8, IL-10, and TNF-α. We suspected that the mechanism involved may be through the inhibition of NF-κB.

Synaptopodin is a biomarker of differentiated podocytes [38], and its expression of synaptopodin gradually increases during nephrogenesis and podocyte differentiation. Synaptopodin is involved in the formation of foot processes along with actin filaments [40], and it plays a significant role in the development and maintenance of these structures. Interestingly, both undifferentiated and differentiated podocytes express WT-1, another podocyte biomarker [39]. Podocytes are terminally differentiated cells in mature glomeruli. Cultured immortalized podocytes proliferate at 33 °C and differentiate at 37 °C [41]. As shown in Fig. 1B, podocytes cultured at 37 °C exhibited more spindle-shape branches and intercellular junctions compared with cells at 33 °C (Fig. 1A). Podocytes cultured at 37 °C also had increased expression of synaptopodin (Fig. 1C), indicating that the podocytes used in the following experiments were in a differentiated state.

Early DN is characterized by excessive proteinuria, due to the destruction of SD and foot processes formed by podocytes [42]. Hence, maintaining the structural integrity of SD is expected to be important for preventing or slowing DN. Nephrin is a transmembrane zipper-like protein located at the SD, which guarantees the correct arrangement of the glomerular basement membrane and the podocytes [12, 43]. Mutation of the *NPHS1* gene, which encodes nephrin, has been reported to cause the fusion of foot processes and loss of SD in mice [44]. Similarly, other studies have shown that knock-down of the podocin encoding gene *NPHS2* also led to proteinuria and death of mice, which was associated with the fusion of foot processes and the destruction of SD [10, 45]. Podocin is localized together with nephrin on the membranes to form the filtration barriers. In addition, deletion of the *CD2ap* gene, a cytoplasmic binding protein of nephrin involved in the regulation of the podocyte cytoskeleton and the stabilization of SD, led to the development of proteinuria and fusion of the foot processes in mice [46]. Consequently, nephrin, podocin, and CD2ap were selected as the markers for SD in the current study. Decreased expression of these three genes indicated dysfunction of SD in podocytes. These results are consistent with studies by Saleem et al. [41] and Fukasawa et al. [13]. The beneficial effects of crocin on SD were confirmed through evaluation of nephrin, podocin, and CD2ap at both the mRNA and protein levels.

Oxidative stress is emerging as a critical event and mediator of DN [29]. In diabetes, oxidative damage occurs when there is an imbalance between ROS generation and production of antioxidant enzyme [28]. The IL-8 and IL-10 levels have also been reported to be significantly altered in the early pathogenesis of DN [31, 47]. Consequently, oxidative stress and inflammation are two major factors associated with the pathogenesis of DN [27]. Our results provide evidence that crocin could potentially counteract the oxidation and inflammation induced by high-glucose conditions, because crocin was found to play a protective role in podocytes.

NF- κ B is a crucial nuclear transcription factor that is closely involved in the regulation of the inflammatory response [48]. NF- κ B is bound within the cytoplasm via interactions with the I κ B family of inhibitory proteins. When cells receive harmful stimuli, the I κ B proteins are immediately phosphorylated [36], resulting in exposure of nuclear localization signals on the NF- κ B subunits. The sequestered NF- κ B is freed and translocates to the nucleus, where it induces the production of pro-inflammatory factors, such as TNF- α and IL-1 β [49]. As described above, I κ B α is an endogenous inhibitor of NF- κ B, whereas p-I κ B α has the ability to activate NF- κ B. In this study, p-I κ B α expression was notably inhibited by crocin and PDTC, as shown in Fig. 5A and B, indicating that both were both able to suppress NF- κ B. As reported previously, IL-1 β and TNF- α are the target genes for NF- κ B [50]. Hence, these two inflammatory factors were further studied to investigate the role of NF- κ B in podocytes under diabetic conditions, as shown in Fig. 5C and D. The results showed that PDTC reinforced the inhibitory effects of crocin on the glucose-induced expression of IL-1 β and TNF- α , suggesting that crocin prevented podocyte injury through the regulation of NF- κ B. Taken together, our results demonstrate that crocin prevents high glucose-induced oxidation and inflammation of podocytes through inhibition of NF- κ B, providing a new therapeutic strategy for DN and associated podocyte injuries.

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Disclosure Statement

The authors declare that they have no conflict of interest.

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