Quercetin suppresses NF-κB and MCP-1 expression in a high glucose-induced human mesangial cell proliferation model

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Abstract. Diabetic nephropathy (DN), which is characterized by mesangial cell proliferation, is a common complication observed in diabetic patients. The protective effects of quercetin for DN have been reported; however, the mechanisms have yet to be determined. We aimed to identify the underlying mechanism for quercetin protection against DN. High glucose (HG)-induced human mesangial cell (HMC) proliferation, a feature of the early stages of diabetic nephropathy, was employed as an in vitro model. Cells were grown in normal glucose (5.6 mM), high glucose (30 mM) or high glucose with various concentrations of quercetin. Cell proliferation, cell cycle progression, and expression of NF-κB and MCP-1 were examined by MTT assay, DNA staining, immunocytochemistry and western blot analysis, respectively. Additionally, the role of NF-κB in HMCs cultured in high glucose had significantly greater proliferation, accumulation in the G1 phase, upregulated NF-κB and MCP-1 expression. Quercetin treatment reversed the effects of high glucose in a dose-dependent manner. Cotreatment of quercetin with pyrrolidine dithiocarbamate (PDTC), an inhibitor of NF-κB activation, suggest that the effects of quercetin are partially mediated by NF-κB signaling. Quercetin partially suppresses the effects of high glucose in HMC cultures, which are mediated at least in part through the suppression of NF-κB.

Introduction

Diabetes mellitus is widely considered to be an inflammatory disease (1), and diabetic neuropathy (DN) is a commonly observed serious complication. Human mesangial cell (HMC) proliferation is a histological feature of DN; it is also observed in renal diseases, causing loss of renal function and subsequent failure (2). However, the precise cellular mechanisms governing HMC hyperproliferation remain unclear although diverse stimuli, including growth factors, inflammatory mediators, and metabolic abnormalities such as hyperglycemia, are known to induce HMC proliferation (3).

Quercetin is a flavonoid that naturally occurs in plants in various glycosidic forms and has potentially beneficial effects in numerous medical conditions (4). Quercetin interacts with phosphoinositide 3-kinases (5), a family of enzymes that regulate cell growth, proliferation, and differentiation; they are also involved in the pathogenesis of diabetes mellitus (6,7). Studies in animal and/or cell models have also indicated that quercetin possesses anti-inflammatory properties and quenches free radicals (8,9). Protective effects of quercetin in diabetes mellitus and DN have also been reported in rat models (10-12). In addition, the protective effects of quercetin have been observed in a streptozotocin (STZ)-induced model of diabetes mellitus (11-14). Furthermore, Kim et al (15) reported that dietary quercetin attenuated fasting and postprandial hyperglycemia. In portal hypertensive rats, quercetin blocked NF-κB signaling, thereby reducing the production of compounds that mediate the pathogenesis of portal hypertensive gastropathy (16). These studies indicate a theoretical basis for the clinical use of quercetin to prevent and treat DN; however, the mechanisms mediating its protective effects have yet to be determined.

The objective of the study was to identify the underlying mechanism for quercetin protection against DN, the most commonly observed serious complication of diabetes mellitus with a feature of hyperproliferation of mesangial cell (HMC) at the early stages. Therefore, HMCs cultured in high glucose were used to mimic hyperglycemia. HMC proliferation, cell cycle progression were examined by MTT assay and DNA staining, respectively. Additionally, the role of NF-κB was explored by examining its expression as well as its downstream target, MCP-1, which is a putative inflammatory mediator that is upregulated in diabetes and other inflammatory processes.

Key words: diabetic nephropathy, human mesangial cells, nuclear factor-κB, quercetin, chemoattractant protein-1

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Abbreviations: ANOVA, analysis of variance; DN, diabetic nephropathy; HG, high glucose; HMC, human mesangial cell; NG, normal glucose, NF-κB, nuclear factor-κB; PKC, protein kinase C; PDTC, pyrrolidine dithiocarbamate; ROS, reactive oxygen species; QU, quercetin; SDs, standard deviations; STZ, streptozotocin; MTT, tetrazolium blue
Materials and methods

Cell culture and treatment. HMCs were kindly provided by Professor Changlin Mei (Department of Nephrology, Shanghai Changzheng Hospital). Cells were cultured in low glucose-containing DMEM medium (Gibco, Carlsbad, CA) supplemented with 10% fetal calf serum (Jiangbin Biotechnology Co., Ltd., Hangzhou, China), 105 U/l penicillin (Wuhan Second Pharmaceutical Factory, Wuhan, China), and 10 g/l streptomycin (Dalian Pharmaceutical Factory, Dalian, China) at 37˚C (5% CO2) in a humidified incubator (Model 2300, Shel-Lab Co., USA). Under these conditions, the cells maintain with the morphologic and functional characteristics of normal HMCs. After the cells attained 90% confluence, they split and counted using 0.25% trypsin and a Neubauer hemocytometer. Cells were cultured at a density of 1x10^4/ml in 25 cm^2 culture flasks. After 80% adherence, the cells were then cultured in low-glucose serum-free DMEM medium for 24 h for synchronization.

The following treatment groups were used in the present study: i) NG (normal glucose, 5.6 mM); ii) HG (high glucose, 30 mM); iii) HG + 0.1% DMSO (dimethyl sulfoxide, the National Institute for the Control of Pharmaceutical and Biological Products, China); iv) HG+QU (quercetin, ranging from 0.5x10^-3 to 4x10^-3 M); v) HG+PDTC (100 µM) (Sigma, St. Louis, MO); vi) HG+QU+PDTC. Quercetin was obtained from the National Institute for the Control of Pharmaceutical and Biological Products. Relevant indicators were measured for all the groups at 12, 24 and 48 h in three independent experiments.

Cell proliferation (MTT) assay. The cells were seeded at a final cell density of 1x10^4 in 96-well plates after which 200 µl of 0.4% calf serum medium was added to each well for 24 h. The cells were synchronized in the stationary phase. Six replication wells for each concentration were used. Cell proliferation was determined by the addition of 20 µl of 5 g/l tetrazolium blue (MTT) solution (Fluka Co., USA.) to each well. After 4 h, the crystals were fully dissolved, a plate reader (Model 680; Bio-Rad, Hercules, CA) was used to measure the absorbance of each well at 490 nm with reference at 530 nm. The proliferation and inhibition rates were calculated using the following equations: proliferation rate = (ODhigh glucose group - ODnormal glucose group) / ODnormal glucose group x 100/100; inhibition rate = (ODhigh glucose group - ODdrug group) / ODhigh glucose group x 100/100.

Cell cycle analysis. Cells (1x10^6) were collected by centrifugation (250 x g for 5 min). After the supernatant was removed without disturbing the pellet, 1 ml of ice-cold 70% EtOH was added dropwise to the cell pellet with vortexing. Cells were stored at -20°C until DNA staining. One milliliter of DNA staining buffer was added to the pellets for 15 min before acquisition on the flow cytometer. Data were analyzed by software.

Subcellular fractionation. Cells in log-phase growth were cultured in the following treatment groups: i) NG, ii) HG, iii) HG+PDTC (100 µM PDTC), iv) HG+QU (4x10^-3 M), and v) HG+QU+PDTC. Cells were collected at 12, 24 and 48 h after treatment to observe the nuclear translocation of NF-κB and MCP-1. Cells were washed with cold PBS, after which 400 µl of cold cytoplasmic lysis buffer A (DMSO). The cells were incubated on ice for 30 min. The lysed cells were then scraped, and the cell lysate was transferred to an Eppendorf tube containing 1 ml of 10% NP-40. The tube was strongly agitated in an oscillator for 10 sec at 10,000 x g (4°C). The supernatant (i.e., the cytoplasmic fraction) was collected after centrifugation for 5 min and stored at -70°C. To obtain the nuclear fraction, 500 µl cold nuclear lysate B was used to resuspend the pellet. After agitation for 30 min followed by centrifugation for 10 min at 14,000 x g at 4°C, the supernatant (i.e., the nuclear protein extract) was collected and stored at -70°C. Protein samples were quantified using a Protein Assay kit (Pierce, Rockford, IL).

Immunocytochemistry. The cells (1x10^5/ml) were seeded onto sterile slides in 12-well culture plates for 24 h. After 75% cells re-attached, the cells were synchronized using serum-free DMEM medium. After 24 h, the culture medium was replaced with DMEM medium containing 5% serum for another 24 h culture based on the experimental group requirements after which they were separated into the following treatment groups: i) NG, ii) HG, iii) HG+PDTC (100 µM PDTC), iv) HG+QU (4x10^-3 M), and v) HG+QU+PDTC. At 12, 24 and 48 h after treatment, immunohistochemical analysis was undertaken to observe the nuclear translocation of NF-κB and MCP-1.

The cells on the coverslips were washed with 0.01 M PBS three times, fixed with 4% paraformaldehyde for 20 min, and washed again with PBS. The coverslips were then fan-dried at room temperature and mounted with neutral resin. After PBS washing three times for 5 min, the cells were permeabilized with 0.5% Triton X-100 (Xiamen Tagene Biotechnology Co., Xiamen, China) for 20 min, and washed three times with PBS for 5 min. Endogenous peroxidase was inactivated using 3% H2O2 for 15 min. After three more PBS washes for 5 min each, the cells were incubated in 5% BSA blocking solution for 15 min at 37°C. Cells were next incubated in either mouse anti-human NF-κB (1:100) or rabbit anti-human MCP-1 (1:50) primary antibodies in a humidified chamber overnight at 4°C. After PBS washing three times for 5 min, PV-9000 two-step reagents (Zhongshan Goldenbridge Biotechnology Co., Ltd., Beijing) 1 and 2 were added dropwise sequentially and incubated for 20 and 30 min at 37°C, respectively. After PBS washing three times for 5 min, DAB developing was performed using DAB development kit (Zhongshan Goldenbridge Biotechnology Co., Ltd.), according to the manufacturer’s instructions. The slides were rinsed thoroughly with distilled water prior to hematoxylin staining for 15-30 sec and gradient alcohol dehydration. The slides were mounted and observed under a microscope (Nikon AFS-DX, Japan).

Cells containing yellow-brown particles in more than 25% of the cytoplasm and nucleus were defined as positive. The densest areas of positive NF-κB p65 and MCP-1 cells were selected separately for counting under x200 magnification; four areas of each treatment in triplicate were analyzed. Image-Plus 5.0 software was used to analyze the ratio of positive cells.

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(17); pyrrolidine dithiocarbamate (PDTC), an inhibitor of NF-κB activation, was also employed. These studies may form the basis for the development of potential therapies to treat hyperglycemia-induced DN.
Western blot analysis. Cells were cultured in 25 cm² flasks and underwent the following treatments: i) NG, ii) HG, iii) HG+PDTC (100 µM PDTC), iv) HG+QU (4x10⁻⁵ M), and v) HG+QU+PDTC. All cells were collected in logarithmic phase growth. After washing with cold PBS, 400 µl cold lysis buffer A was added for 30 min with occasional mixing. Cells were harvested by scraping, and lysates were obtained by centrifugation at 10,000 x g for 5 min at 4°C. Supernatants were collected and stored at -70°C until use. Protein concentrations were analysed using the Protein Assay kit (Pierce) following the manufacturer’s instructions. Samples (25 ml) were separated on 10% SDS-PAGE followed by electrotransferred to PVDF membrane (Pierce) as per the manufacturer’s instructions. The PVDF membranes were incubated for 2-4 h at room temperature or at 37°C after the addition of 0.1 ml/cm² blocking solution containing 10% skim milk powder (Boster Bioengineering Co., Ltd., Wuhan, China) and 0.05% Tween-20 (Sigma) TBST. The membranes were incubated overnight at 4°C with the following primary antibodies diluted in the blocking solution containing 5% skim milk powder and 0.05% TBST: NF-κB p65 (1:400; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), MCP-1 (1:200; Boster Bioengineering Co.), and β-actin (1:500; Santa Cruz Biotechnology, Inc.). After the membranes were rinsed with TBS containing 0.05% Tween-20 three times for 10 min, they were incubated with the appropriate secondary antibody (HRP-conjugated goat anti-rabbit and goat anti-mouse antibodies; Santa Cruz Biotechnology, Inc.) diluted in the blocking solution at 1:5,000 for 2 h at room temperature. The membranes were collected and rinsed with TBS containing 0.05% Tween-20 three times for 10 min after which they were incubated with an enhanced chemiluminescence reagent (Zhongshan Goldenbridge Biotechnology Co., Ltd.) and exposed to X-OMAT BT medical X-ray film (Kodak). The bands were analyzed using Quantity One software. NF-κB p65 or MCP-1 protein expression was normalized to the housekeeping gene, β-actin.

Statistical analysis. The results were expressed as means and standard deviations (SDs). The comparison of means among the six treatment groups was performed by one way analysis of variance (ANOVA). When a significant difference was found, multiple comparisons were conducted by the Bonferroni procedure with type-I error adjustment. The level of significance was set at 0.05. Statistical analyses were performed using SAS 9.1 statistical software (SAS Institute Inc., Cary, NC).

Results

High glucose-cultured HMCs successfully represent an in vitro DN model. High glucose-cultured HMCs were used to mimic hyperglycemia in this study since hyperproliferation of HMCs was featured at the early stages of DN. Gross morphology examination revealed that HMCs cultured in normal glucose (NG) media were spindle-shaped, irregular star-shaped, or triangular, with mostly oval, dark nuclei, and numerous short or long cytoplasmic protrusions (Fig. 1A). Upon culturing in high glucose (HG) medium, the cells became hypertrophic and had altered morphology. To ensure the high glucose (30 mM) condition in HMCs appropriately represent an in vitro DN model, cell proliferation was examined by MTT assay. Cell proliferation significantly increased in high glucose-cultured HMCs as compared to the cells cultured with normal glucose (5.6 mM) at 12, 24 and 48 h (P<0.001) (Fig. 1B). The cell proliferation in the HG group increased by 62.04, 41.63 and 32.66% at 12, 24 and 48 h, respectively. These data indicate that high glucose-cultured HMCs mimic the physiology observed in DN with the characteristics of HMC hyperproliferation.
Effects of quercetin on morphology and proliferation in high glucose-cultured HMCs. Protective effects of quercetin in diabetes mellitus and DN have also been reported in rat models (12-14). Our data showed that quercetin treatment at 40 µM led to smaller cells with irregular shapes, larger gaps between adjacent cells, and weakened cell adhesion (Fig. 1A). To examine the influence of quercetin on HG-induced HMC hyperproliferation, quercetin of various concentrations were added to the HMC cultures in the presence of high glucose. There was no difference in cell proliferation between the HG alone and vehicle control groups (HG+DMSO) at 12, 24 and 48 h (Fig. 1C). However, quercetin exhibited a significant inhibitory effect on HG-induced HMC proliferation at 12, 24 and 48 h in a dose-dependent manner (all P<0.001).

Effects of high glucose and quercetin on cell cycle progression. Since quercetin exerted an inhibitory effect on high glucose-induced HMC proliferation, we next examined whether quercetin affects cell cycle progression that results in decreased cell proliferation. High glucose significantly increased the percentage of cells in G1 phase (75.73±0.59%) as compared to the NG group (P<0.05) (Fig. 2). High glucose-cultured HMC presented a dose-dependent decrease in percentages of G1 cells in response to quercetin treatment. HMC cultured in high glucose exhibited a significant decrease in the percentage of G2 cells as compared to the NG group (8.8±3.2 vs. 27.43±5.5%). Treatment of quercetin at various concentrations in HG-cultured HMCs showed a trend towards increasing the percentages of G2 cells, however, no significant difference was observed.

High glucose-induced spatial and temporal expression of NF-κB and MCP-2. It has been reported that dietary quercetin attenuated fasting and postprandial hyperglycemia (15) and quercetin was proven to block NF-κB signaling in a portal hypertensive rat model (16). The expression patterns of NF-κB and its known downstream inflammatory factor MCP-1 were thus determined in the absence or presence of high glucose. It
is well known that activated NF-κB translocates from the cytoplasm into the nucleus. We examined the spatial and temporal expression of NF-κB in HMC cultured under NG or HG conditions. The HMC under high glucose condition exhibited not only increased protein levels of NF-κB but also altered distribution, NF-κB translocated from the cytoplasm to nucleus (Fig. 3A). Expression of MCP-1, one of the prominent downstream factors in NF-κB signaling, was simultaneously examined and the data showed that MCP-1 expression was increased in response to HG within 48 h while suppressing it after 72 h (20,21). However, the exact mechanism governing these effects remains to be determined.

Effects of quercetin on HG-induced NF-κB and MCP-2 protein expression. The effect of quercetin on HG-induced NF-κB activity in HMC was examined. Fig. 4A shows a representative image of western blotting. HMCs cultured under NG condition exhibited basal levels of NF-κB and MCP-1 expressions, while high glucose condition significantly increased NF-κB and MCP-1 expression (P<0.05) (Fig. 4). Quercetin treatment, which has an inhibitory effect on HG-induced HMC proliferation, was observed to suppress both NF-κB and MCP-1 expression significantly as compared to the HG group (P<0.01). PDTC, a putative inhibitor of NF-κB activity, was introduced and acted as expected to inhibit NF-κB activation induced by HG condition. Either quercetin or PDTC suppressed HG-induced NF-κB and MCP-1 expression; however, both contributed partially to the suppression of expression. Combined treatment of quercetin and PDTC exhibited additive effect on inhibition of NF-κB and MCP-1 expression, which was significantly lower than either treatment alone (P<0.01) and almost at the basal level as compared to the NG group.

The effects of high glucose and quercetin on NF-κB and MCP-1 expression were further confirmed using immunocytochemistry (Fig. 5). Representative images are shown in Fig. 5A for all treatment groups. The basal level expression of NF-κB and MCP-1 was observed in the NG group and was significantly increased upon culturing in HG condition (P<0.05). The expression of NF-κB and MCP-1 significantly decreased in the quercetin and PDTC groups. Cotreatment with QU+PDTC further reduced the expression of both NF-κB and MCP-1 (P<0.001).

**Discussion**

Because a role for inflammation in the pathogenesis of DN has increasingly been observed (18), it has been suggested that quercetin's anti-inflammatory properties may mediate its protective effects against DN. The objective of this study was to identify the underlying mechanism for quercetin protection against DN. HMCs cultured in high glucose had significantly greater proliferation, accumulation in the G1 phase, upregulated NF-κB and MCP-1 expression. These effects were reversed with quercetin treatment in a dose-dependent manner.

Culturing HMCs in high glucose was used as a model to mimic their proliferation observed in the early stages of DN and resulting pathological cause of renal dysfunction. In the present study, increased HMC proliferation was observed upon culturing in high glucose-containing medium, which is consistent with the results from previous studies (18,19). Additionally, increased MC proliferation with high glucose treatment associated with decreased p21 and increased CDK4 and CDK2 activity (19), which was reflected in the present study as hyperproliferation of HMCs and an increased percentage of HMCs in G1 phase were observed upon culture in high glucose condition.

Studies have shown that high in vitro glucose serves a dual function on HMC growth, stimulating proliferation within 48 h while suppressing it after 72 h (20,21). However, the exact mechanism governing these effects remains to be determined. Chronic exposure of HMCs to HG may induce apoptotic signaling, possibly resulting in microvascular injury to the diabetic kidney (22). Because we wanted to determine the effects of quercetin on DN, characterized by HMC hyperproliferation, all studies were assessed at 48 h culture in high glucose-containing medium.

Quercetin significantly inhibited the proliferation of high glucose-induced HMCs in a dose-dependent manner. Because of QU's anti-inflammatory functions, its effects on NF-κB expression, a transcription factor that regulates the expression of cytokines, adhesion factors, cytokine receptors, chemokine receptors, and anti-apoptotic proteins (23), was also assessed. Previous studies have reported that high glucose can activate NF-κB in HMCs through protein kinase C (PKC) and reactive oxygen species (ROS) signaling, stimulating proliferation of HMCs and the secretion of...
inflammatory mediators, such as MCP-1. In the present study, high glucose treatment induced expression of NF-κB as well as its downstream target, MCP-1, which is similar to that reported in previous studies (24,25). In addition, quercetin decreased both NF-κB and MCP-1 expression, which was similar to that observed in mast cells and endothelial cell, respectively (26,27). Furthermore, additive effects of cotreatment with quercetin and PDTC, an inhibitor of NF-κB activity, were observed.

MCP-1 induces macrophage infiltration in kidney tissues, clears glycosylated albumin and oxidized-LDL, and reduces renal tissue damage (28); its expression increases in the renal tissues of DN patients. Its local overexpression could prolong the inflammatory state, inducing renal tissue damage (29). High blood glucose may induce MCP-1 expression through the p38 MAPK signaling in endothelial cells and glomerular MCs; it also directly stimulates MCP-1 mRNA and protein expression in glomerular MCs (30). In type 2 DM patients, plasma MCP-1 expression was significantly higher than that in healthy volunteers, and it was also positively correlated to urinary albumin excretion (31). Chacón et al (32) reported increased MCP-1 mRNA expression in the adipose tissues of patients with type 2 DM.

In the present study, PDTC was used as a specific inhibitor as control to compare with the effectiveness of quercetin. It is a specific NF-κB inhibitor that prevents IκB phosphorylation, reducing IκB degradation as well as decreasing lipid peroxidase activity recent studies have confirmed that PDTC can induce tumor cell apoptosis.

Quercetin and its derivatives are the most widely distributed flavonoids in the plant kingdom without obvious side effects. They have many pharmacological activities, mainly including anti-oxidant, anti-inflammatory, free radical scavenging, blood pressure lowering, anti-cancer, anti-aging, anti-mutagenic, and anti-atherosclerosis effects (16,17). However, whether quercetin can inhibit NF-κB signaling has yet to be determined. Recent studies have shown that ROS, as an important stimulator of NF-κB activation, and plays a key role in DN pathogenesis (6). Therefore, we speculated that the protective effects of quercetin may be a result of blocking the ROS-NF-κB inflammatory pathway. In the present study, quercetin inhibited the expression of NF-κB and MCP-1 in HMCs, suggesting that its anti-inflammatory function may block the development of diabetic glomerular inflammation. Although this provides a theoretical basis to further understand and evaluate the role of quercetin in the prevention and treatment of diabetic nephropathy, further in vivo studies are necessary.

The present study has limitations that warrant discussion. Firstly, the effects of quercetin were only analyzed using in vitro studies. Therefore, analysis of the effects of quercetin in an animal model of DN is required. Furthermore, although these results showed that quercetin has, to some extent, an inhibitory effect on the NF-κB signaling pathway, the mechanism by which it inhibits NF-κB activation and the specific functions of its target genes in DN requires further investigation. In addition, its effects on ROS were not explored.

In conclusion, in summary, high glucose stimulates HMC proliferation as well as the expression of NF-κB p65 and MCP-1, which may further induce structural damage of glomerular MCs and thereby alter kidney function. Quercetin reduces HMC proliferation in response to high glucose and reduces NF-κB p65 and MCP-1 expression. Thus, its anti-inflammatory activity may be responsible for its protective effects observed in diabetic kidneys.

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References


