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Morin inhibits cell proliferation and fibronectin accumulation in rat glomerular mesangial cells cultured under high glucose condition

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ABSTRACT

Morin, is a natural bioflavonoid isolated from Chinese herbs of the Moraceae family, has been reported to possess antidiabetic activity. However, the role of morin on glomerular mesangial cells (MCs) proliferation and extracellular matrix (ECM) accumulation in diabetic condition is still unclear. Therefore, in this study, we investigated the role of morin on cell proliferation and ECM accumulation in rat glomerular MCs cultured under high glucose (HG) condition. Our results showed that morin inhibited HG-induced MC proliferation, arrested HG-induced cell-cycle progression, reversed HG-inhibited expression of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$. It also inhibited HG-induced ECM expression, ROS generation and NOX4 expression in MCs. Furthermore, morin suppressed HG-induced phosphorylation of p38 MAPK and JNK1/2 in MCs. These data suggest that morin inhibits HG-induced MC proliferation and ECM expression through suppressing the activation of p38 MAPK and JNK signaling pathways. Thus, morin may be useful for the prevention or treatment of diabetic nephropathy.

1. Introduction

Diabetic nephropathy, one of the most complications of diabetes mellitus, is a major cause of end-stage renal disease [1]. Glomerular mesangial cells (MCs) play important roles in the physiological and pathological processes of diabetic nephropathy [2]. MCs proliferation and accumulation of extracellular matrix (ECM) are hallmarks of diabetic nephropathy [3]. Furthermore, emerging studies have indicated that hyperglycemia is involved in the overproduction of ECM in mesangial cells [4]. Thus, it is important to effectively blocking MC proliferation and ECM accumulation may prevent the development and progression of diabetic nephropathy.

Morin, is a natural bioflavonoid isolated from Chinese herbs of the Moraceae family, has been reported to possess many biological activities including antioxidant, anti-inflammatory, neuro-protection, antidiabetic, and anti-carcinogenic activities [5–7]. It was reported that morin hydrate ameliorated cisplatin-induced renal injury by suppressing oxidative stress and inflammation in mice [8]. Morin was found to inhibit growth and invasion of the highly metastatic breast cancer cells at least in part via the inactiv ation of Akt pathway [9]. In addition, Vanitha et al. found that morin treatment significantly preserved the normal histological appearance of pancreatic islets as well as to preserve insulin-positive β-cells in streptozotocin-rats [10]. Another study confirmed that morin administration reduced blood glucose, serum insulin, leptin, malondialdehyde, interleukin-6, and monocyte chemoattractant protein-1 (MCP-1) levels and increased serum adiponectin levels in a high-fat-diet-induced obese mice [11]. However, the role of morin on MC proliferation and ECM accumulation in diabetic condition is still unclear. Therefore, in this study, we investigated the role of morin on cell proliferation and ECM accumulation in rat glomerular MCs cultured under high glucose (HG) condition.

2. Materials and methods

2.1. Cell culture

The study was approved by the Institutional Animal Care and Use Committee of the Second Affiliated Hospital of Zhengzhou University (China). Rat MCs was isolated from young SD rat kidneys according to the method described previously [12]. Cells were cultured in Dulbecco's modified eagle's medium (DMEM)

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supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. Passages between 3 and 8 were used in all experiments.

At sub-confluence, mesangial cells were incubated with serum-free DMEM medium for 24 h. Then, the cells were stimulated with various concentrations of morin (25 and 50 μM; Sigma Chemical Co., St. Louis, MO, USA) with or without 30 mM D-glucose for further 24 h.

2.2. Cell proliferation assay

Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. In brief, after treatment, 20 μl of MTT (5 mg/ml; Sigma Chemical Co., St. Louis, MO, USA) was added to each well and incubation continued at 37 °C for 4 h. Then, 150 μl of DMSO (Sigma Chemical Co., St. Louis, MO, USA) was added into each well and incubated again for 10 min under gentle shaking at 37 °C to dissolve the tetrazolium dye. The optical density was measured spectrophotometrically at 570 nm using an ELISA microplate reader (Invitrogen).

2.3. Cell cycle assay

The cell-cycle progression was evaluated using flow cytometry. In brief, after treatment, the MCs were fixed with pre-cooled 70% ethanol at 4 °C overnight and incubated with 1 mg/ml RNase A (Takara Biotechnology, Dalian, China) for 30 min at 37 °C. Then, cells were added propidium iodide (50 μg/ml; Sigma, St. Louis, MO, USA) at 4 °C for 30 min. The cell cycle was analyzed by flow cytometry assay.

2.4. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Total RNA was isolated from MCs using TRIzol® reagent (Life Technologies, Inc., Rockville, MD, USA) according to the manufacturer's instructions. 5 μg RNA was reverse-transcribed into cDNA using Maloney-murine leukemia virus reverse transcriptase (Clontech Laboratories, Inc., Palo Alto, CA, USA). The primers of each gene were shown as follows: fibronectin forward, 5'-AGAGCAAGCCTGAGCCTGAAG-3' and reverse, 5'-TCGCCAATCTTG-TAGGACTGACC-3'; type IV collagen forward, 5'-ATGCCCTTTCTCTTCTGCAA-3' and reverse, 5'-GAAGGAATAGCC-GATCCACA-3'; β-actin forward, 5'-GAGGCA CTCTTCCAGCCTTC-3' and reverse, 5'-GGATGTCCACGTCACACTTC-3'. The PCR procedure was as follows: 94 °C for 3 min; 94 °C for 20 s, 59 °C for 30 s, and 72 °C for 25 s; 2 s for plate reading for 40 cycles; and melting curve from 65 to 95 °C. The mRNAs were normalized to β-actin mRNA with the comparative Ct method.

2.5. Western blot analysis

The cells were homogenized and lysed with RIPA lysis buffer (100 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% Triton X-100, 1 mM EDTA, 10 mM β-glycerophosphate, 2 mM sodium vanadate and protease inhibitor). The protein concentration was determined using the BCA assay. The protein lysates (40 μg/lane) were subjected to 10% SDS-PAGE and electrophoretically transferred to Immobilon P Millipore (Bedford, MA, USA). The membrane was blocked with 2% non-fat dry milk in Tris-buffered saline (TBS) for 1 h, followed by incubation with primary antibodies (dilution, 1:1000) targeting p21, p27, fibronectin, type IV collagen, NOX4, p-p38, p38, p-JNK1/2, JNK1/2 and GAPDH (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4° C. Subsequently, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:1000 in the blocking buffer at room temperature for 1 h. Finally, bound proteins were visualized by enhanced chemiluminescence (ECL) detection system (Amersham, Little Chalfont, UK).

2.6. Evaluation of reactive oxygen species (ROS)

2',7'-dichlorodi-hydrofluorescein diacetate (DCFH-DA) was used to detect intracellular ROS level. Briefly, after treatment, MCs were washed with PBS, and then incubated with 10 mM DCFH-DA in the dark for 30 min at 37 °C. Finally, the cells were observed under a fluorescence microscope. The fluorescence intensity was measured by a fluorospectrophotometer at excitation/emission maxima of 485/525 nm.

2.7. Statistical analysis

Data are expressed as the mean ± standard deviation. Statistical significance was analyzed with the one-way factorial ANOVA or the Student's two-tailed *t*-test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Morin inhibits HG-induced MC proliferation

First, we measured the effect of morin MC proliferation by MTT assay. As shown in Fig. 1A, the proliferation of MCs was not altered by morin treatment under normal glucose. In addition, we observed that HG dramatically promoted MC proliferation, as compared with normal glucose group. However, morin treatment inhibited HG-induced MC proliferation in a concentration-dependent manner (Fig. 1B).

3.2. Morin arrests HG-induced cell-cycle progression in MCs

The next experiment was performed to investigate the effect of morin on cell-cycle progression in high glucose-induced MCs. As expected, high glucose significantly decreased the cell proportion in G0/G1 phase, as well as increased the cell proportion in S phase, as compared with normal glucose group. However, morin treatment significantly increased G0/G1 phase cells, and decreased S phase cells in a concentration-dependent manner, as compared with HG-treated cells (Fig. 2A). Furthermore, we investigated the effect of morin on cell regulatory proteins expression in MCs induced by HG. As indicated in Fig. 2B, HG obviously reduced the protein expression levels of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$, as compared with normal glucose group. However, morin treatment greatly reversed HG-inhibited the protein expression levels of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ in MCs.

3.3. Morin inhibits HG-induced ECM expression in MCs

Various studies have demonstrated that the accumulation of glomerular ECM proteins is involved the development of diabetic nephropathy. Thus, we measured the effect of morin on ECM proteins expression in MCs induced by HG. The results of RT-qPCR analysis demonstrated that the mRNA expression levels of fibronectin and type IV collagen was markedly increased by HG, compared with NG control. Strikingly, morin obviously reversed HG-induced the mRNA expression levels of fibronectin and type IV collagen in MCs (Fig. 3A). In addition, Western blot analysis showed that morin significantly reversed HG-induced the protein expression levels of fibronectin and type IV collagen in MCs (Fig. 3B).