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

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ABSTRACT

Glomerular mesangial cells (MCs) proliferation and extracellular matrix (ECM) accumulation have been recognized as major pathogenic events in the progression of diabetic nephropathy. Betulinic acid (BA), (3β-hydroxy-lup-20(29)-en-28-oic acid), is a naturally occurring pentacyclic lupane group triterpenoid, and it has been shown to possess glucose-lowering property. However, the role of BA on MC proliferation and ECM accumulation in diabetic condition remains unclear. So, in the present study, we investigated the role of BA on cell proliferation and ECM accumulation in rat glomerular MCs cultured under high glucose (HG) condition. In the current study, we demonstrated that BA suppressed HG-induced MC proliferation, arrested HG-induced cell-cycle progression, reversed HG-inhibited expression of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$. It also suppressed HG-induced fibronectin (FN) expression in MCs. Furthermore, BA inhibited HG-induced phosphorylation of ERK1/2 and p38MAPK in MCs. In conclusion, our present study demonstrated that BA inhibited HG-induced cell proliferation and FN expression in MCs via inhibiting ERK1/2 and p38MAPK pathways. Thus, BA may serve as a potential drug for the treatment of diabetic nephropathy.

1. Introduction

Diabetic nephropathy is one of the most serious microvascular complications of diabetes and the leading cause of end-stage renal failure, which leads to high morbidity and mortality rates in diabetic patients [1]. It is characterized by mesangial cell (MC) proliferation and accumulation of extracellular matrix (ECM) components such as fibronectin (FN) [2]. There is increasing evidence that high glucose (HG) is one of the major factors in the development of diabetic nephropathy [3–5]. High glucose can stimulate MC proliferation and hypertrophy, increase synthesis of IV collagen protein, and lead to renal glomerulus mesangial matrix proliferation [6]. Therefore, it is important to effectively block MC proliferation and ECM accumulation in order to prevent the development and progression of diabetic nephropathy.

Pentacyclic triterpenoids including the oleanane, ursane and lupane groups are widely distributed in many medicinal plants, which are commonly used in traditional medicine for the treatment of diabetes and diabetic complications [7]. Betulinic acid (BA), (3β-hydroxy-lup-20(29)-en-28-oic acid), is a naturally occurring pentacyclic lupane group triterpenoid. BA has been demonstrated to possess a number of biological properties such as an anti-cancer [8], cardioprotective [9], anti-inflammatory [10] and anti-HIV properties [11]. More recently, Kim et al. reported that BA significantly inhibited the hepatic glucose production (HGP) and gene expression levels of PGC-1α, PEPCK, and G6Pase in high fat diet-fed mice [12]. However, the role of BA on MC proliferation and ECM accumulation in diabetic condition remains unclear. So, in the present study, we investigated the role of BA on cell proliferation and FN accumulation in rat glomerular MCs cultured under HG condition.

2. Materials and methods

2.1. Chemicals and reagents

Betulinic acid, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DMSO, propidium iodide (PI) fluorescent reagent were purchased from Sigma Chemicals Co., St. Louis, USA. RPMI-1640 medium and MEM medium were from Gibco Invitrogen Corporation (Carlsbad, CA).

2.2. Cell culture

Isolation of mice mesangial cells (MMCs) was performed according to the method described previously [13]. Cells were

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cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and were kept in a humidified incubator that was maintained at 37 °C and supplied with 5% CO_2 and 95% air. Passages between 3 and 8 were used in all experiments.

At subconfluence, mesangial cells were incubated with serum-free MEM medium for 24 h and then divided into six groups as follows: (a) control, where cells were kept in MEM medium without FBS, containing normal glucose concentration of 5.6 mM (NG group); (b) high glucose, where cells were cultured in MEM medium without FBS containing 30 mM D-glucose (HG group); (c) low-dose BA group (20 μM); and (d) high-dose BA group (40 μM) in 30 mM D-glucose condition. Cells were exposed to above conditions for 24 h.

2.3. Cell proliferation assay

The MTT assay was used to measure cell proliferation. In brief, MCs were seeded into 96-well plates at 1000 cells per well and then incubated with NG or HG in the presence or absence of BA for 24 h. Then 20 μl of MTT (5 mg/ml) was added to each well and incubation continued at 37 °C for 4 h. The medium was then carefully removed and DMSO was added into each well and the cell viability was determined by measuring the absorbance at 570 nm using an ELISA microplate reader (Invitrogen). The cells incubated in control medium were considered as 100% viable.

2.4. Cell cycle assay

Cell cycle analysis was performed using flow cytometry. After 24 h treatment with BA, cells were harvested and fixed in 70% ethanol and stored at −20 °C. Followed by washed twice with phosphate buffered saline (PBS), cells were harvested by trypsinization, centrifuged, and suspended with 1 ml cold PBS and then fixed in methanol for 30 min on ice. Fixed cells were washed with PBS twice, then incubated in RNAse solution (100 μg/ml) for 30 min at 37 °C. Subsequently, the cells were incubated in propidium iodide (PI) solution (100 μg/ml in PBS) at room temperature for 30 min. The cell cycle was detected by flow cytometry (Invitrogen).

2.4.1. Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated from MCs using TRIzol® reagent (Life Technologies, Inc., Rockville, MD, USA) according to the manufacturer's instructions. Approximately 5 μg total RNA for each sample were reverse transcribed into first strand cDNA. The levels of gene mRNA transcripts were analyzed by using the specific primers and SYBR Green I reagent and the RT-PCR kit, according to the manufacturer's instructions, on Bio-Rad iQ5 Quantitative PCR System (Takara, China). The specific primers for FN were forward, 5′-AGAGCAAGCCTGAGCCTGAAG-3′ and reverse, 5′-TCGCCAATCTTGTAGGACTGACC-3′; β-actin forward, 5′-GAGGCA CTCTTCCAGCCTTC-3′ and reverse, 5′-GGATGTCCACGTCACACTTC-3′. The PCR procedure was as follows: 94 °C for 4 min; 94 °C for 20 s, 55 °C for 30 s, and 72 °C for 20 s; 2 s for plate reading for 35 cycles; and melting curve from 65 to 95 °C. β-actin was used as a control for normalizing gene expression. The data obtained were calculated by $2^{-\Delta\Delta Ct}$ and treated for statistical analysis as described previously [14].

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 in the lysates was determined by BCA protein assay kit (Beyotime, Nantong, China). The proteins (30 mg/lane) were subjected to 10% SDS-PAGE and electrophoretically transferred to Immobilon P Millipore (Bedford, MA, USA). Non-specific binding was blocked by incubating with 5% non-fat milk in PBS with Tween 20 buffer at room temperature for 1 h. Then, the membrane was incubated overnight at 4 °C with primary antibodies (dilution, 1:1000) targeting p21, p27, FN, p-p38, p38 p-ERK1/2, ERK1/2 and β-actin (all from Santa Cruz Biotechnology, CA, USA), followed by incubation with the corresponding secondary antibodies at room temperature for 1 h. Finally, the proteins were detected using the standard enhanced chemiluminescence ECL method using a kit purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

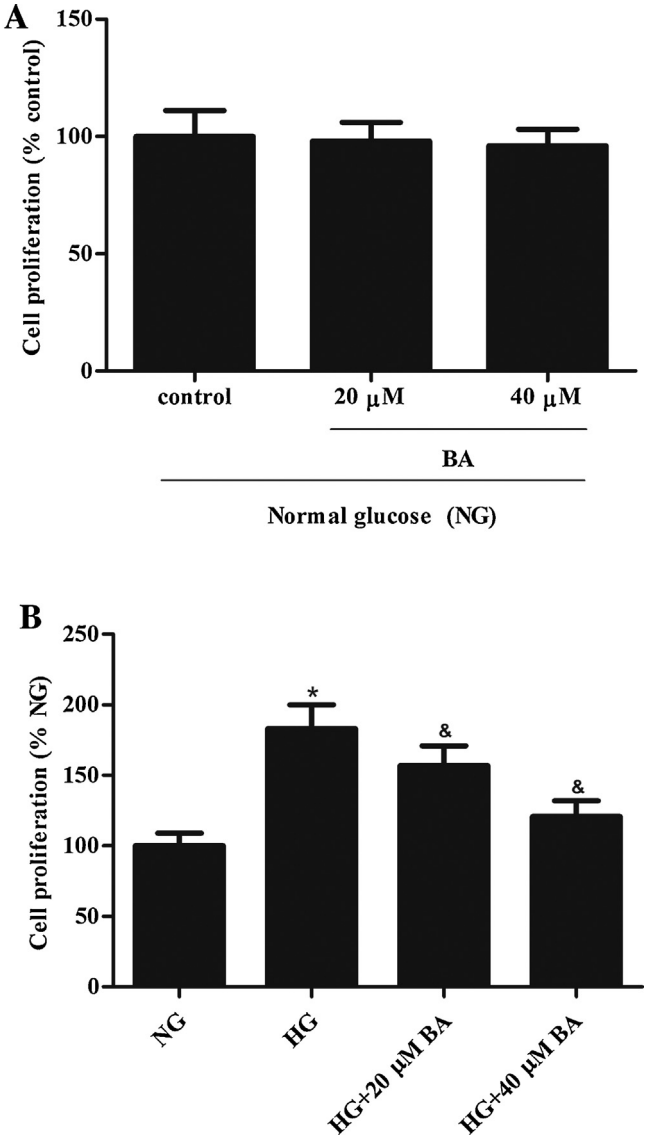

Fig. 1. Effects of betulinic acid on cell proliferation induced by high glucose in MCs. (A) Cells were pretreated with and without BA (20, 40 μM) for 30 min before incubation with 5.6 mM NG for 24 h. Cell proliferation was determined by MTT assay. (B) Cells were pretreated with and without BA (20, 40 μM) for 30 min before incubation with 30 mM HG for 24 h. Cell proliferation was determined by MTT assay. BA treatment inhibited HG-induced MC proliferation in a concentration-dependent manner. Data represent means ± SD. of three experiments. $^{*}P < 0.05$ vs. NG group, & $P < 0.05$ vs. HG group.