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Effect of magnolol on TGF-β1 and fibronectin expression in human retinal pigment epithelial cells under diabetic conditions

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Abstract

Magnolol, a natural product isolated from *Magnolia officinalis*, has various pharmacological effects, such inhibition of effect on inflammation and tumor metastasis, protection against cerebral ischaemic injury, and potent antioxidant activity. In this present study, we evaluated the inhibitory effects of magnolol on transforming growth factor-β1 (TGF-β1) and fibronectin expression induced by high concentrations of glucose or S100b (a specific receptor of advance glycation end products ligand) in human retinal pigment epithelial cells (human RPE cells). No effect on cell growth was found with magnolol (up to 20 μg/ml) using a colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide (MTT) assay. High glucose (25 mM) or S100b (5 μg/ml) induced increases in expression of TGF-β1 and fibronectin. The increases in TGF-β1 and fibronectin expression with high glucose or S100b were prevented by magnolol in a dose-dependent manner. Also, magnolol inhibited extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK)/Akt activation. The present study demonstrates that high glucose- or S100b-induced TGF-β1 and fibronectin expression, but this increased expression is inhibited by magnolol *via* the ERK/MAPK/Akt signaling pathway in human RPE cells.

Keywords: Diabetic retinopathy; TGF-β1; Fibronectin; Retinal pigment epithelial cells; Magnolol; High glucose; S100b

1. Introduction

Hyperglycemia has an important role in the pathogenesis of diabetic complications by increasing the amount of advanced glycation end products (Gugliucci, 2000; Ahmed, 2005). Advanced glycation end products stimulate expression of extracellular matrix proteins such as fibronectin, laminin, and collagen IV mediated by transforming growth factor (TGF)-β in diabetic complication such as diabetic renal and vascular disease (Kim et al., 2001; Li et al., 2004; Wolf, 2004). Increased synthesis of extracellular matrix proteins contributes to the development of vascular basement membrane thickening, which is common in diabetic retinopathy (Roy et al., 2003).

In mammals, three isoforms of TGF-β (TGF-β1, -β2, and -β3) are known to be involved in the proliferation, migration, differentiation, apoptosis, and accumulation of extracellular matrix proteins in various cells types (Massague and Chen, 2000). TGF-β has been identified as a critical mediator and regulator in pathophysiological processes of ocular tissue development or repair (Border and Noble, 1994; Gordon-Thomson et al., 1998; Lee and Joo, 1999; Saika, 2006). TGF-β induction of vascular endothelial growth factor (VEGF) secretion by retinal pigment epithelial (RPE) cells has an important role in neovascularization in diabetic eye disease (Nagineni et al., 2003). RPE cells located between the neurosensory retina and the vascular choroids form the outer blood retinal barrier and have a key role in the pathological processes that lead to loss of vision (Spilsbury et al., 2000; Schwesinger et al., 2001).

Magnolol (5,5′-diallyl-2,2′-dihydroxybiphenyl), a component of *Magnolia officinalis*, has several pharmacological activities, such as an antidepressant effect, an inhibition of tumor metastases, protection against cerebral ischaemic injury, an anti-platelet effect, and an antioxidant effect (Nakazawa et al., 2003; Ikeda et al., 2003; Chang et al., 2003; Pyo et al., 2002; Lo et al., 1994). In addition, *M. officinalis* is used for the

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treatment of diabetes and diabetic complications in Korean traditional herbal medicines and prescriptions (Hur, 1999). We have previously reported that an ethanolic extract of Magnoliae cortex had an *in vitro* inhibitory effect on the formation of advanced glycation end products, which have important roles in the development of diabetic complications (Kim et al., 2002). Furthermore, magnolol inhibits advanced glycation end products formation and sorbitol accumulation in streptozotocin-induced diabetic rats and prevents the development of diabetic nephropathy in type 2 diabetic Goto-Kakizaki rats (Kim et al., 2002; Lee et al., 2006; Sohn et al., 2007). In this study, we have examined the pharmacological effects of magnolol on TGF-β1 and fibronectin expression, inhibition of oxidative stress, and specific signaling pathways in human RPE cells cultured under diabetic conditions.

2. Materials and methods

2.1. Materials

S100b protein (bovine brain) was obtained from Calbiochem (EMD Biosciences, Inc. San Diego, CA, USA). Reverse transcriptase (RT) and polymerase chain reaction (PCR) kits were from Bioneer (Daejeon, South Korea). Anti-phospho-p38, anti-p38, anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-Akt, and anti-Akt were from Cell Signaling (Beverly, MA, USA). Human TGF-β1 ELISA systems were obtained from R&D systems (Minneapolis, MN, USA). The following reagents were purchased from the venders indicated: Dulbecco's modified eagle's medium (DMEM)/F-12, and fetal calf serum (FBS, Gibco BRL, Grand Island, NY, USA), and enhanced chemiluminescence reagent (ECL, Amersham Biosciences, UK). All other reagents were from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Isolation of magnolol from Magnoliae cortex

The herbal medicine Magnoliae cortex (cortex of *M. officinalis* Rehder; Magnoliaceae) was obtained from the Baekje herbal medicine store (Daejeon, South Korea) and identified by Professor J.-H. Kim, Division of Life Science, Daejeon University. Cortex of *M. officinalis* Rehder has been deposited at the herbarium of the Department of Herbal Pharmaceutical Development, Korea Institute of Oriental Medicine (No. 1240). We used a recrystallization method that did not require column chromatography for mass isolation of magnolol (5,5′-diallyl-2,2′-dihydroxybiphenyl) (Fig. 1) from the root barks of *M. officinalis*. Briefly, the powdered plant materials (5 kg) were extracted with 80% EtOH (30 L) for one week at room temperature, concentrated with a rotary evaporator, and lyophilized, and the entire procedure was repeated for four times. Distilled water and *n*-hexane were added and the *n*-hexane layer was separated. The *n*-hexane layer was concentrated and magnolol (60 g) was isolated from the *n*-hexane layer directly by recrystallization (*n*-hexane/$CHCl_3$). The isolated magnolol was identified by comparing the nuclear magnetic resonance (NMR) data obtained with those of published values (Bang et al., 2000) and by thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC) analysis with the standard compound (Wako Pure Chemical Industries, Japan).

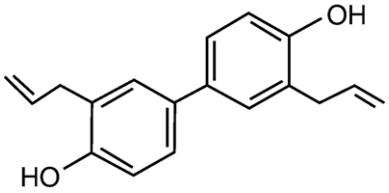

Fig. 1. Structure of magnolol.

2.3. Cell culture and experimental conditions

Human retinal pigment epithelial (RPE) cells, ARPE-19, were obtained from the American Type Culture Collection (ATCC, #CRL-2302, Rockwille, MD) and were maintained in continuous culture at 37 °C/5% CO_2 using DMEM/F-12 containing 10% heat-inactivated FBS, 1000 U ml^{-1} penicillin and streptomycin, 3 mM L-glutamine and non-essential amino aids. Cells were plated into 6-well culture dishes and used for experiments when they reached 80% confluence. Fresh serum-free media were placed on the cells 24 h before experiments.

2.4. MTT cell viability assay

The cytotoxicity of magnolol was determined by a colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide (MTT) assay. Briefly, the human RPE cells were placed at density of 3×10^3 cells per well and were grown in a final volume of 100 μl media per well in 96-well plates. The cells were grown in the presence of magnolol at concentrations ranging from 1 to 100 μg/ml at 37 °C in the 5% CO_2 incubator. After adding 10 μl of MTT labeling reagent containing 5 mg/ml MTT in phosphate-buffered saline (PBS) to each well, plates were incubated for 4 h. Solubilization solution (100 μl) containing 10% sodium dodecyl sulfate (SDS) in 0.01 M HCl was added to each well and the wells were incubated for another 24 h. The absorbance was then measured with a microtiter plate reader (Bio-Tek, Winooski, VT, USA) at a test wavelength of 550 nm with a reference wavelength of 690 nm. The optical density (OD) was calculated as the difference between the absorbance at the reference wavelength and that observed at the test wavelength. Percentage viability was calculated as (OD of treated sample / OD of untreated control) × 100.

2.5. Cell harvesting and Western blot analysis

Human RPE cells were seeded in 6-well dishes at initial concentrations of 10^5 cells/well and incubated until 80% confluence was reached. Growth media were removed and the cells were incubated for 24 h in serum-free media. The cells were then incubated with or without a high concentration of glucose (HG, 25 mM) or S100b (5 μg/ml) for 24 h. The cells were harvested and protein concentrations for each sample were