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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).

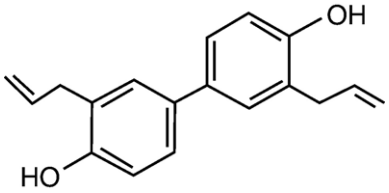

Fig. 1. Structure of magnolol.

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

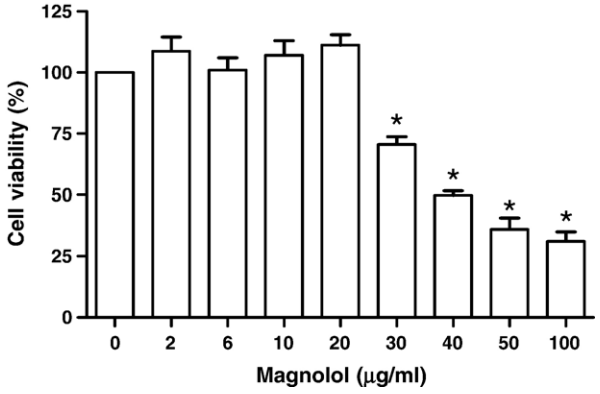

Fig. 2. Effect of magnolol in the viability of human RPE cells. Human RPE cells were incubated in the presence of the indicated magnolol concentrations for 24 h. Cells were stained with MTT and analyzed with a microtiter plate reader. The experiments were repeated four times. The results are presented as the means±S.E.M. *$P<0.001$ *vs.* untreated cells.

determined using the bicinchoninic acid assay. Protein samples (50 μg) were separated by electrophoresis on 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose, as described previously (Kim et al., 2005). Each nitrocellulose blot was probed using the specified primary antibody and incubated with the secondary anti-rabbit or anti-mouse horseradish peroxidase (HRP)-conjugated IgG. The chemiluminescent (ECL plus Western detection reagents) was applied to the nitrocellulose for 5 min, after which the film was exposed (for between 15 s and 5 min depending on signal strength). Protein bands were visualized and quantified using Scion Image analysis software (NIH Image, USA).

2.6. Quantikine TGF-β1 immunoassays (ELISA assay)

Human RPE cells were plated in 6-well culture dishes and incubated in DMEM/F-12 with 10%FBS. After 80% confluence was reached, the medium was replaced with the serum-free medium containing magnolol for 24 h under high glucose or S100b treatment. Medium supernatant fractions were harvested. Following activation of TGF-β1 by treatment with 1 N HCl (0.1 ml/ 0.5 ml of conditioned media) for 10 min at room temperature, 0.1 ml 1.2 N NaOH/0.5 M HEPES was added. Quantikine human TGF-β1 ELISA was carried out according to the manufacturer's protocol, and levels were normalized to total protein. Medium alone without cells incubated under the same conditions used as blank control for the ELISA.

2.7. RNA isolation and relative reverse transcription-polymerase chain reaction (RT-PCR)

Human RPE cells grown to confluence in 6-well culture dishes were incubated in serum free medium for 24 h. After treatment at the indicated concentrations, total RNA was isolated using TRI reagent (MRC, Cincinnati, OH) according to the manufacturer's manual and cDNA was synthesized with 1 μg of RNA using AccuPower RT premix and random hexamers (Bioneer, Daejeon, Korea). Human TGF-β1, fibronectin, and β-actin mRNA were amplified using the following primers, TGF-β1 5′-TAT GCC GCC CTC CGG GCT-3′ (sense), 5′-AGT CGA CGT GAA CGT CCT C-3′ (anti-sense), fibronectin 5′-TAC GCA AAT GGT TCA GCC CC-3′ (sense), 5′-AGC AGC ATG ATC AAA ACA CTT C-3′ (anti-sense), and β-actin 5′-ATG GAT GAT ATC ATC GCC GC-3′ (sense), 5′-TGA CCA CGG ACC CCG CGG-3′ (anti-sense) using a Takara PCR Thermal Cycler (Japan). β-actin primers were used as the internal control. The sequences for the TGF-β1 mRNA primers included: forward and reverse sequences. For each experiment, a parallel control PCR was performed with primers for β-actin. PCR was performed for 34 cycles for TGF-β1 preceded by determination of the linear increase in PCR that occurred between 25 and 38 cycles. PCR was performed for 22 cycles for β-actin

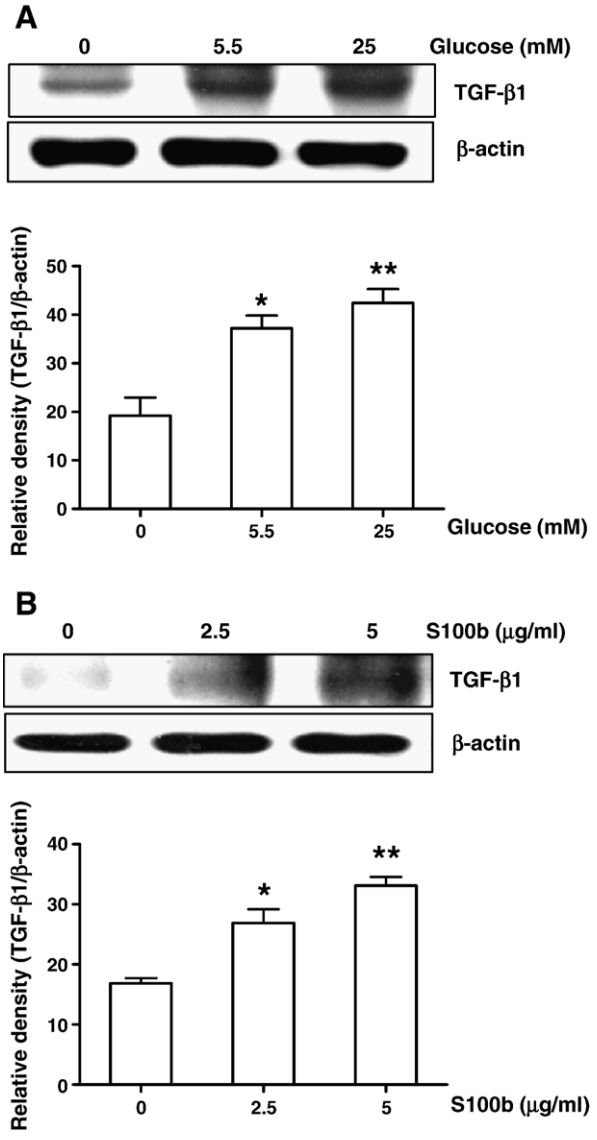

Fig. 3. High glucose or S100b increases TGF-β1 protein expression in human RPE cells. Cells were incubated with glucose (A) or S100b (B) and TGF-β1 protein (39 kDa) levels were detected by Western blot. Data are presented as means±S.E.M. of three experiments. *$P<0.05$, **$P<0.01$ *vs.* untreated cells, respectively.

preceded by determination of the linear increase in PCR that occurred between 20 and 25 cycles. The PCR product was loaded onto 1.2% agarose gels along with DNA markers in a separate lane (Fermentas 1 kb Generuler), stained with ethidium bromide, and visualized with UV light.

2.8. Determination of lipid peroxidation

The formed malondialdehyde (MDA, a product of lipid peroxidation) levels were determined as described previously (Lee et al., 1996). In brief, isolated cell lysates were homogenized in 1 ml of Tris–Cl buffer having pH 7.4 using a polytron homogenizer. An aliquot (100 μl) of homogenate was added to each reaction mixture containing 400 μl of 2% SDS, 300 μl of 20% acetic acid, 50 μl of 0.7% thiobarbituric acid, and 60 μl distilled water in a tightly closed caped tubes. Samples were then incubated at 95 °C for 3 h, after incubation samples were cooled to room temperature and then centrifuged at 4000 ×*g* for 5 min. The supernatant was removed and absorbance was taken at 535 nm; quantification was done using the standard curve generated with tetraethoxypropane under similar conditions. Freshly diluted tetramethoxypropane, which yield MDA, was used as a standard. The MDA content was expressed as nmol/mg of protein.

2.9. Statistical analysis

Results are expressed as means±S.E.M. of multiple experiments. Paired Student's *t*-tests were used to compare two groups, or ANOVA with Tukey's multiple comparison tests using PRISM software (Graph Pad, San Diego, CA). A *P* value <0.05 was considered to be statistically significant.

3. Results

3.1. Effects of magnolol in the viability of human RPE cells

To assess the effect of magnolol (Fig. 1) on the viability of human RPE cells, cells were cultured with magnolol at final concentration of 0–100 μg/ml for 24 h and the MTT assay was performed. Cells cultured in magnolol-free media were used as the control. The viabilities of cells incubated with magnolol at

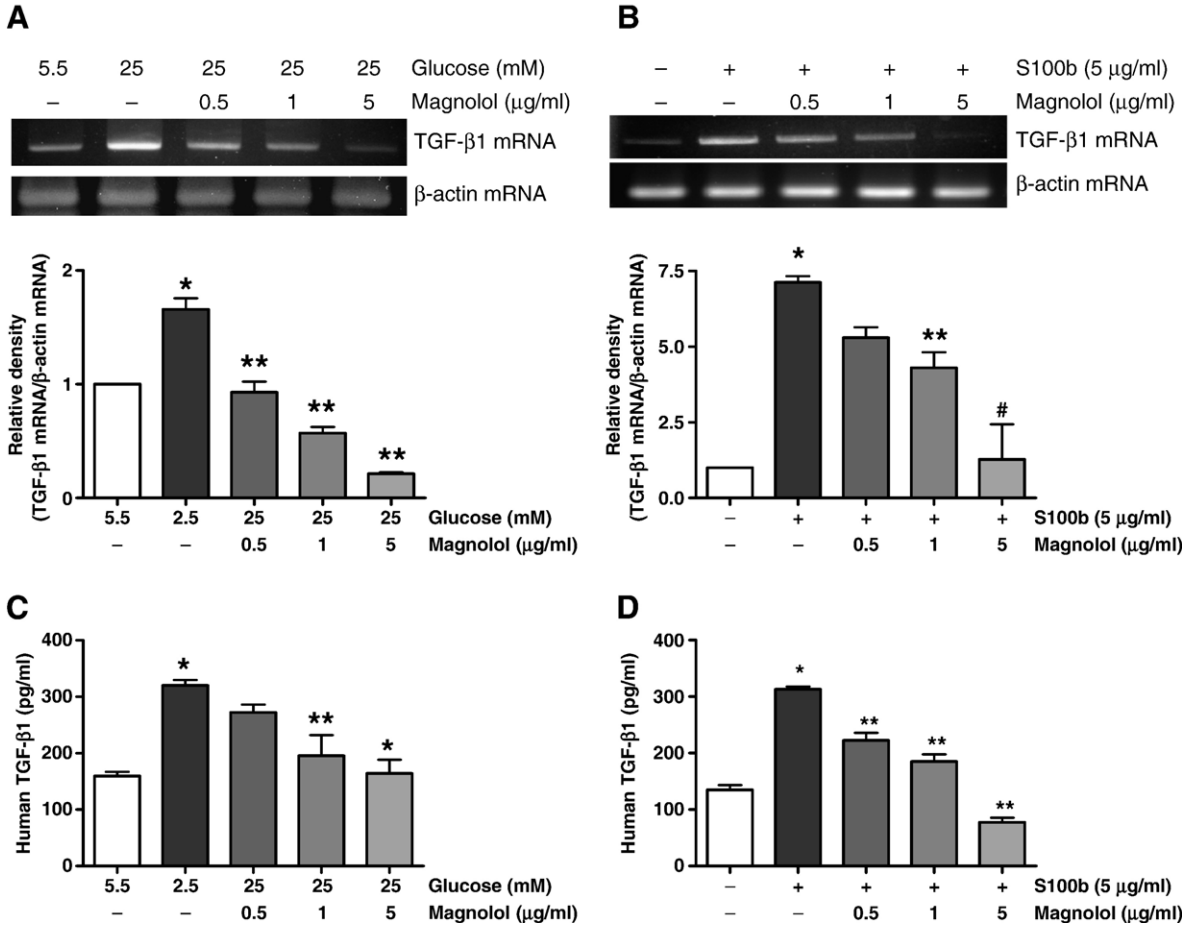

Fig. 4. Inhibitory effects of magnolol on high glucose or S100b-induced TGF-β1 mRNA expression and protein secretion. TGF-β1 mRNA expression analyzed by RT-PCR was increased with high glucose (25 mM, A) or S100b (5 μg/ml, B) and suppressed in a dose-dependent manner with magnolol (0.5~5 μg/ml). High glucose (C) or S100b (D)-induced TGF-β1 secretion was analyzed by ELISA. Data are presented as means±S.E.M. of three experiments. (A) **P*<0.001 *vs.* 5.5 mM glucose; ***P*<0.01 *vs.* cells treated with high glucose. (B) **P*<0.001 *vs.* untreated cells; ***P*<0.05, #*P*<0.001 *vs.* cells treated with S100b, respectively. (C) **P*<0.01 *vs.* 5.5 mM glucose; ***P*<0.05, **P*<0.01 *vs.* cells treated with high glucose, respectively. (D) **P*<0.001 *vs.* untreated cells; ***P*<0.001 *vs.* cells treated with S100b.

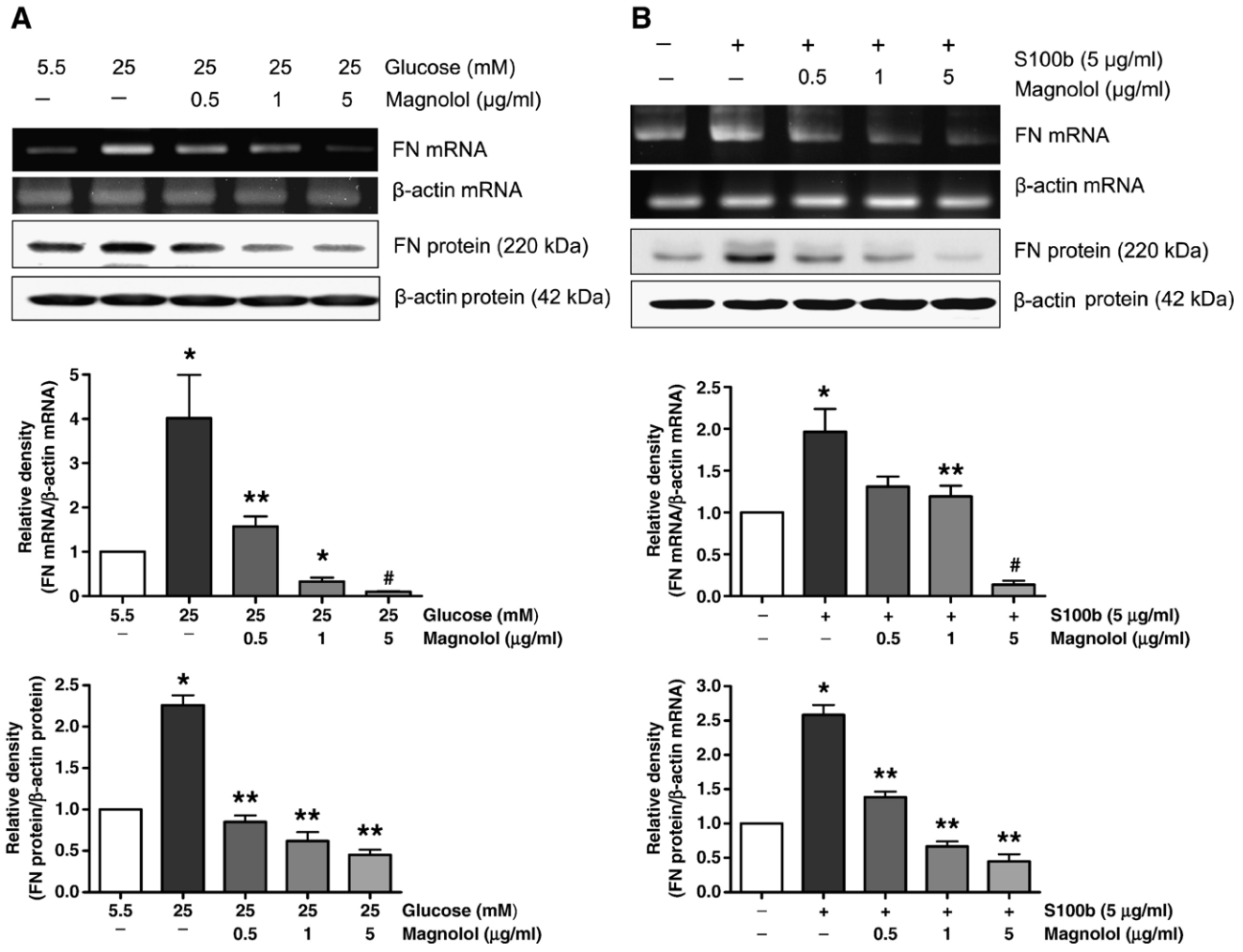

Fig. 5. Inhibitory effects of magnolol on high glucose or S100b-induced fibronectin mRNA and protein. Fibronectin mRNA and protein were increased with high glucose (25 mM, A) or S100b (5 μg/ml, B) and suppressed in a dose-dependent manner by magnolol (0.5–5 μg/ml). Data are presented as means±S.E.M. of three experiments. (A) Fibronectin mRNA; *$P<0.01$ *vs.* 5.5 mM glucose, **$P<0.05$, *$P<0.01$, #$P<0.001$ *vs.* cells treated with high glucose, respectively. Fibronectin protein; *$P<0.001$ *vs.* 5.5 mM glucose, **$P<0.001$ *vs.* cells treated with high glucose (B) *$P<0.01$ *vs.* untreated cells; **$P<0.05$, #$P<0.001$ *vs.* cells treated with S100b, respectively. Fibronectin protein; *$P<0.001$ *vs.* untreated cells; **$P<0.001$ *vs.* cells treated with S100b.

concentrations of 2 μg/ml, 4 μg/ml, 6 μg/ml, 10 μg/ml, 20 μg/ml, 30 μg/ml, 50 μg/ml and 100 μg/ml for 24 h were 108.7±5.9%, 101.0±5.0%, 107.1±5.9%, 111.2±4.3%, 70.6±3.1%, 49.8±1.9%, 31.1±3.9%, and 35.9±4.7% of the control value, respectively (Fig. 2). These data suggest that magnolol exerted no significant alternation in the viability of RPE cells until it was at a concentration of 20 μg/ml.

3.2. Expression of TGF-β1 protein in human RPE cells under diabetic conditions

Studies have shown that hyperglycemic conditions upregulate TGF-β1 levels in pericytes and microvascular endothelial cells (Khan et al., 2004). To examine the effects of high glucose or S100b (a specific receptor of advance glycation end products ligand) on TGF-β1 protein expression in human RPE cells, normal glucose (5.5 mM) and high glucose (25 mM) concentrations or S100b (2.5 μg/ml and 5 μg/ml) were exposed for 24 h and the TGF-β1 levels were observed by Western blot. Results in total human RPE cells are shown in Fig. 3A. TGF-β1 expression was significantly increased with at 0, 5.5, and 25 mM of glucose. Compared with control, S100b also increased TGF-β1 protein in a dose-dependent manner (Fig. 3B). These results indicate that TGF-β1 protein expression is induced in human RPE cells under diabetic conditions.

3.3. Magnolol inhibits TGF-β1 mRNA expression and protein secretion in human RPE cells under diabetic conditions

To determine whether magnolol can inhibit high glucose or S100b-induced TGF-β1 expression by human RPE cells, we measured levels of TGF-β1 in human RPE cells by RT-PCR. In human RPE cells cultured in high glucose or S100b, TGF-β1 levels were significantly increased by 70%, whereas magnolol treatment showed decrease in TGF-β1 compared to high glucose or S100b treated human RPE cells (Fig. 4A and B). Our RT-PCR analysis showed significant increases in TGF-β1 mRNA in RPE cells treated with high glucose or S100b. We

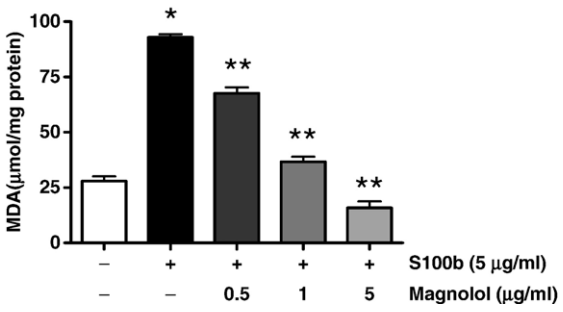

Fig. 6. Inhibitory effect of magnolol on malondialdehyde production in S100b-induced RPE cells. MDA levels were measured in S100b-induced human RPE cells treated with magnolol for 24 h. Data represent means±S.E.M. of at least three experiments performed in triplicate. *$P<0.01$ *vs.* untreated cells; **$P<0.05$ *vs.* S100b-treated cells.

therefore evaluated whether the TGF-β1 mRNA levels were also reduced in magnolol-treated RPE cells (Fig. 4A and B). For analysis of TGF-β1 proteins, ELISA was carried out using supernatant from control cells and cells grown with high glucose or S100b with or without magnolol pretreatment. Human RPE cells were treated with various doses of magnolol (0.5–5 μg/ml) for 1 h and then stimulated with high glucose (25 mM) or S100b (5 μg/ml) for 24 h. This pretreatment with magnolol clearly inhibited high glucose- or S100b-induced TGF-β1 protein secretion in a dose dependent manner (Fig. 4C and D). These findings first reported that high glucose- or S100b-induced TGF-β1 mRNA and protein expression and secretion inhibited by magnolol in human RPE cells.

3.4. Magnolol inhibits expression of fibronectin in human RPE cells under diabetic conditions

TGF-β has direct effects on extracellular matrix protein synthesis (Mauviel, 2005). Several lines of evidence indicate that increased deposition of extracellular matrix proteins such as fibronectin plays a key role in the pathogenesis of eye diseases such as diabetic retinopathy (Roy et al., 2003; Stitt et al., 2002). To determine whether magnolol can inhibit high glucose- or S100b-induced fibronectin expression in human RPE cells, we measured levels of fibronectin mRNA and protein in human RPE cells. In human RPE cells cultured under high glucose or S100b conditions, the fibronectin mRNA levels were significantly increased by 4-fold and 1.9-fold, respectively. By contrast, magnolol pretreatment reduced the fibronectin mRNA levels in a dose-dependent manner compared with the levels observed in high glucose- or S100b-treated human RPE cells (Fig. 5A and B). This result is the first report that high glucose- or S100b-induced fibronectin mRNA and protein expression can be inhibited by magnolol in human RPE cells.

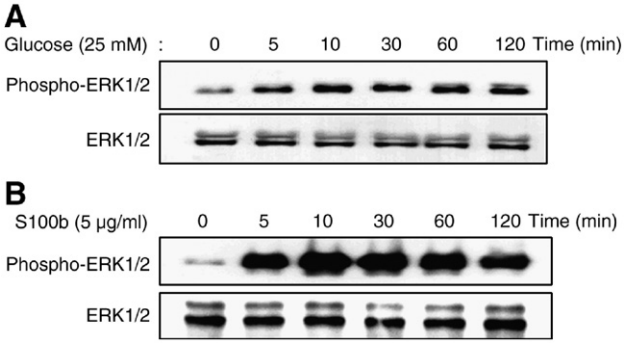

Fig. 7. Activation of ERK1/2 by high glucose or S100b in human RPE cells. Human RPE cells were stimulated with high glucose (25 mM, A) or 5 μg/ml S100b (B) for the indicated periods of time (minutes), and cell lysates were immunoblotted with anti-phospho-ERK1/2 (44/42 kDa, upper panel) and anti-ERK1/2 (lower panel) antibodies.

3.5. Effect of magnolol on lipid peroxidation

Advanced glycation end products form by way of sequential glycation and oxidation reactions; advanced glycation end products can accumulate as a consequence of oxidation reactions alone. Magnolol has an inhibitory effect on lipid peroxidation in rat hepatic mitochondria (Chiu et al., 1999). In order to find out if the magnolol pretreatment reduces S100b-induced lipid peroxidation, lipid peroxidation was estimated by

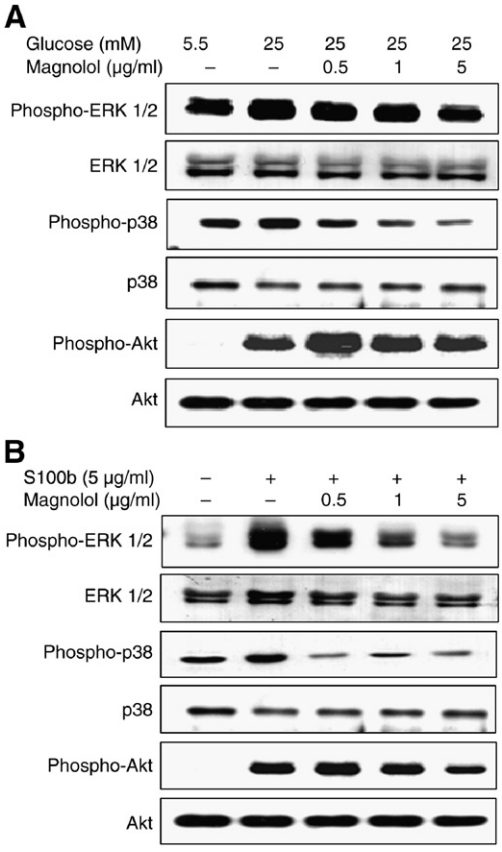

Fig. 8. Inactivation of ERK1/2, p38 MAPK, pAkt by magnolol in human RPE cells under high glucose or S100b. Total cell lysates of RPE cells, pretreated with magnolol, were prepared after treatment with high glucose (A) or S100b (B) for 10 min. Proteins separated by SDS-PAGE were immunoblotted and probed with antibodies to phosphorylated forms of pERK1/2, p38 MAPK or pAkt, or to ERK, total p38 MAPK, or Akt as an internal control. Probing with the anti-phospho antibodies demonstrated increasing phosphorylation of ERK1/2 and p38MAPK after high glucose (*lane-2* in A) or S100b (*lane-2* in B) treatment. Pretreatment of magnolol inhibits high glucose and S100b induced phosphorylation of ERK, p38MAPK, and Akt.

measuring the formed malondialdehyde (MDA) colorimetrically, as described previously (Lee et al., 1996). MDA level in S100b-induced RPE cells was significantly ($P<0.05$) increased as compared with control (92.93±1.33 *vs.* 27.94±2.22 μmol/mg of protein). Treatment with magnolol inhibited this increase in MDA levels in S100b-treated RPE cells (Fig. 6).

3.6. Magnolol inhibits high glucose- or S100b-induced ERK/MAPK/Akt signaling in human RPE cells

To obtain evidence for the activation of extracellular signal-regulated kinase (ERK) 1/2 with high glucose or S100b, we assessed the phosphorylation levels of ERK1/2. Treatment with high glucose or S100b resulted in a transient phosphorylation of ERK1/2 in a time-dependent manner. The levels of the phosphorylated ERK, ERK1 and ERK2 increased by 2.2 and 6.7 fold, respectively, at 10 min after high glucose or S100b treatment (Fig. 7). We further tested the effect of magnolol on high glucose- or S100b-induced ERK/mitogen-activated protein kinase (MAPK)/Akt phosphorylation. Pretreatment with magnolol resulted in a dose-dependent inhibition of the increases in TGF-β1 and fibronectin production seen with high glucose or S100b (Figs. 4 and 5). Furthermore, magnolol also significantly blocked S100b-induced phosphorylation of ERK 1/2, p38 MAPK and Akt (Fig. 8B). These data suggest that the ERK/MAPK/Akt signaling pathway is required for magnolol-induced inhibition of TGF-β1 and fibronectin expression.

4. Discussion

In this present study, we have shown that high glucose or S100b stimulate TGF-β1 and fibronectin expression in human RPE cells. Moreover, this study is the first report to show that magnolol inhibits high glucose- or S100b-induced TGF-β1 and fibronectin expression via the ERK/MAPK/Akt-dependent pathway and that it inhibits S100b-induced lipid peroxidation.

Natural products have been used as drugs for various diseases for hundred of years and the ethnobotanical information on medicinal plants lists almost 800 plants that can be used in the control of diabetic mellitus (Alarcon-Aguilara et al., 1998). Several natural compounds and semi-synthetic derivatives have been proposed, discovered, or are currently being used as inhibitors of advanced glycation end products, which prevent the development of diabetic complications in experimental diabetes (Wong et al., 2001; Rahbar and Figarola, 2003; Degenhardt et al., 2002; Stitt et al., 2002; Figarola et al., 2003). The traditional Korean therapeutic system known as Hanbang medicine originated from China, it has been used for hundreds of years safely, and has fewer side-effects compared to pharmacological compounds. *M. officinalis*, one of the medicinal herbs, is used for the treatment of diabetes and diabetic complications in Korean traditional herbal medicines and prescriptions (Hur, 1999).

Several reports indicate that magnolol, a component of *M. officinalis*, has pharmacological activities and, *in vitro*, has a stronger inhibitory effect on advanced glycation end products formation than the effective protein glycation inhibitor, aminoguanidine (Nakazawa et al., 2003; Ikeda et al., 2003; Chang et al., 2003; Pyo et al., 2002; Lo et al., 1994; Kim et al., 2002). In particular, magnolol has potent antioxidant and free-radical-scavenging activities (Lee et al., 2001). S100b-induced lipid peroxidation can be inhibited by magnolol pretreatment in human RPE cells. Reduction of oxidative stress by scavenging superoxides with magnolol may provide an alternative strategy for controlling diabetes. Antioxidants, including ascorbic acid, vitamin E, peroxidase, phenolic antioxidant, or other thiol antioxidants, significantly inhibit TGF-β1 signaling (Lee et al., 2003).

We have found that high glucose increases TGF-β1 and fibronectin expression by activation of the ERK/MAPK/Akt pathway in human RPE cells. In addition, S100b increased TGF-β1 and fibronectin expression *via* the MAPK/ERK/Akt pathway. These increases with high glucose or S100b were reduced in a dose-dependent manner by pretreatment of cells with magnolol. We have clearly demonstrated that magnolol can significantly suppress the expression of TGF-β1 and fibronectin in human RPE cells. Several growth factors have been identified as likely mediators of the effects of high ambient glucose concentrations in diabetic complications, but prominent among these is TGF-β. TGF-β cooperates with VEGF to induce both retinal neovascularization and fibrosis around these new vessels in diabetic complications (Saika, 2006; Ziyadeh, 2004; Nagineni et al., 2003). The concentrations of total and active TGF-β, especially TGF-β2, are also higher in patients with diabetic retinopathy and open-angle glaucoma than in healthy subjects (Ochiai and Ochiai, 2002). Several studies have shown that synthesis of extracellular matrix protein components such as fibronectin, laminin, or collagen IV is upregulated by high glucose or in patients with diabetes, and that these changes are associated with the development of basement membrane thickening, which is common in diabetic retinopathy (Roy et al., 2003). Our results indicate that increased extracellular matrix protein synthesis induced by high glucose or S100b is reduced by magnolol, thereby improving cell monolayer permeability by regulating fibronectin overexpression in human RPE cells. High glucose-induced upregulated fibronectin occurs *via* activation of the MAPK/ERK pathway in endothelial cells, and overexpression of fibronectin is also associated with protein kinase C, NF-kappaB, and AP-1 activation (Xin et al., 2004). Diabetes-induced upregulation of oncofetal fibronectin is dependent on hyperglycemia-induced TGF-β1 and endothelin-1 (Khan et al., 2004). Akt signaling modulates TGF-β activities through direct interaction with Smad3 (Song et al., 2003).

In summary, high glucose or S100b stimulates expression of TGF-β1 and fibronectin *via* specific signaling pathways in human RPE cells. The high glucose- or S100b-induced upregulation of TGF-β1 and fibronectin expression are inhibited by magnolol through inactivation of ERK/MAPK/Akt signaling. Moreover, magnolol inhibits S100b-induced lipid peroxidation. These results reveal a novel role of magnolol that could result in the prevention of the pathologic processes that lead to diabetic retinopathy.

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