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Original Article

Tanshinone IIA protects against methylglyoxal-induced injury in human brain microvascular endothelial cells

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Abstract: Tanshinone IIA is one of the major diterpenes from *Salvia miltiorrhiza Bunge* and has been shown to possess a protective effect on the endothelial cells. The present study aimed to investigate whether tanshinone IIA could protect against methylglyoxal (MGO)-induced injury in human brain microvascular endothelial cells (HBMEC). Using cultured HBMEC, cell viability was measured by MTT assay and trypan blue dye exclusion test. Cellular oxidative stress was measured by production of reactive oxygen species (ROS), thiobarbituric acid reactive substances (TBARS) and H_2O_2. AnnexinV/PI staining and western blot were performed to determine cell apoptosis and protein expression. We found that MGO treatment caused a concentration and time-dependent decrease in cell viability, which was inhibited by pretreatment with tanshinone IIA. Exposure to MGO promoted the accumulation of AGEs, and production of ROS, TBARS and H_2O_2 in the cultured HBMEC, which were inhibited by tanshinone IIA pretreatment. Addition of tanshinone IIA significantly reduced MGO-induced cell apoptosis as shown by flow cytometry. On the molecular level, tanshinone IIA administration altered the expression of apoptosis-related proteins such as p53, Bax, Bcl-2 and cyto C. In addition, MGO treatment remarkably increased the phosphorylation of MAPK family including p38, JNK and ERK. By contrast, addition of tanshinone IIA inhibited the activation of MAPK family members. These data indicated that tanshinone IIA could protect against MGO-induced cell injury through inhibiting MAPK activation in HBMEC.

Keywords: Tanshinone IIA, methylglyoxal, human brain microvascular endothelial cells, apoptosis, MAPK

Introduction

Diabetes is often associated with various micro- and macro-vasculopathies, such as diabetic retinopathy, neuropathy, and systemic atherosclerosis [1, 2]. Cerebrovascular disease remains a leading cause of morbidity and mortality in diabetic patients distinguished by impaired glucose tolerance and poor glycemic control [3].

It has been demonstrated that many factors are involved in the cerebrovascular dysfunction in diabetes. Among them, methylglyoxal (MGO) has been shown to play an important role in the progression of diabetic vascular complications [4, 5]. As one of the most reactive dicarbonyls, MGO has been considered to be an important glycating agent for glycation damage to the mitochondrial proteome [6]. In addition, the cytotoxicity of MGO is mediated by the modification of deoxyribonucleic acid and activation of apoptosis [7]. Recent studies demonstrated that high serum levels of MGO were observed in patients with either type 1 or type 2 diabetes [8]. Moreover, increased exposure to MGO in diabetic patients can induce diabetes-like vascular complications [9]. Therefore, it is important to explore new and effective therapeutic agents to prevent MGO-induced injury in patients with diabetes.

The rhizome of *Salvia miltiorrhiza Bunge* (SM), also known as Tanshen, is an important herb for promoting the circulation of blood and eliminating stasis in Chinese traditional medicine [10-12]. Tanshinone IIA (Tan IIA) is one of the major diterpenes extracted from SM, which could significantly reduce infarct volume and improve neurological deficits of ischemic injury by suppressing oxidative stress and radical-mediated inflammatory insults [13]. In addition,

Tan IIA has been shown to possess an ameliorating effect on the microcirculatory disturbance and target organ injury after ischemia-reperfusion [14]. However, the biological effect of Tan IIA on MGO-induced injury has never been investigated.

In the present study, we tried to investigate the protective function of Tan IIA on human brain microvascular endothelial cells (HBMEC). Our data indicated that Tan IIA could protect against MGO-induced cell injury through inhibiting JNK activation in HBMEC.

Materials and methods

Reagent

Tan IIA was purchased from Sigma (St. Louis, MO, USA)). RPMI-1640 culture medium, fetal bovine serum (FBS), phosphate-buffered saline (PBS), penicillin-streptomycin (PS) and 0.25% (w/v) trypsin/1 mM EDTA were purchased from Gibco (Grand Island, NY, USA).

Cells culture and treatments

HBMEC were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum, 10% Nu-Serum, 5% MEM non-essential amino acids and 5% sodium pyruvate. Cells were treated with indicated concentrations and time points of Tan IIA or MGO. Then, the cells were harvested for further analysis.

Cell viability

Cells viability was evaluated by MTT assay. To be brief, cell density of 3×10^4 (cells/well) was seeded into 96-well plates and left to adhere overnight. The cells were then incubated with or without indicated concentrations of Tan IIA. Then 10 ml of 5 mg/ml MTT was added and incubated in dark at 37°C for 2 h. The absorbance was determined with the wavelength of 570 nm. Than, cell viability was ascertained by trypan blue dye exclusion assay.

ROS content

The amount of ROS was determined by a ROS assay kit (Sigma, USA) according to manufacture's protocol. To be brief, cultured HBMEC were incubated with 2, 7-dichlorofluorescein-diacetate (DCFH-DA) at 37°C for 6 h and washed three times by PBS. Then, cells were placed in wells of a microtiter plate and scanned to visualize the color development.

Thiobarbituric acid reactive substances (TBARS) assay

HBMEC were exposed to the indicated concentrations of Tan IIA or MGO for 24 h. TBARS level was measured using OxiSelect TBARS assay kit (Geneteks Biosciences, Inc.) according to the manufacture's protocol.

Measurement of H_2O_2

Divalent iron ions were oxidized to ferric ions with H_2O_2 and formed a purple product with xylenol orange, which can be used for the detection of H_2O_2. HBMEC were exposed to the indicated concentrations of Tan IIA or MGO for 24 h. The concentration of H_2O_2 released was calculated according to standard concentration curve originated from standard solutions upon the identical experiments.

Flow cytometry analysis

HBMEC were exposed to the indicated concentrations of Tan IIA or MGO for 24 h. The cultured cells were washed with PBS twice and detached with trypsin. Apoptosis cells were detected with annexin V-FITC/PI according to the protocol of Annexin V-FITC cell Apoptosis Detection Kit (BD, USA).

Western blot analysis

After treatment with 30 μg/mL Tan IIA or 200 μM MGO, total cell lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). For Western blotting analysis, primary antibodies including anti-AGEs, p53, Bax, Bcl-2, cyto C, β-actin, p-JNK, t-JNK, p-ERK, t-ERK, p-p38, t-p38 (Cell Signaling, Beverly, MA) were used. Anti-rabbit or anti-mouse secondary antibody conjugated with horseradish peroxidase was also applied (Pierce Chromatography Cartridges, USA). Immunoreactive bands were detected by enhanced chemiluminescence (ECL) kit for Western blotting detection by using a ChemiGenius bioimaging system (SYNGENE, USA).

Statistical analysis

Each experiment was performed in triplicate, and repeated at least three times. All the data

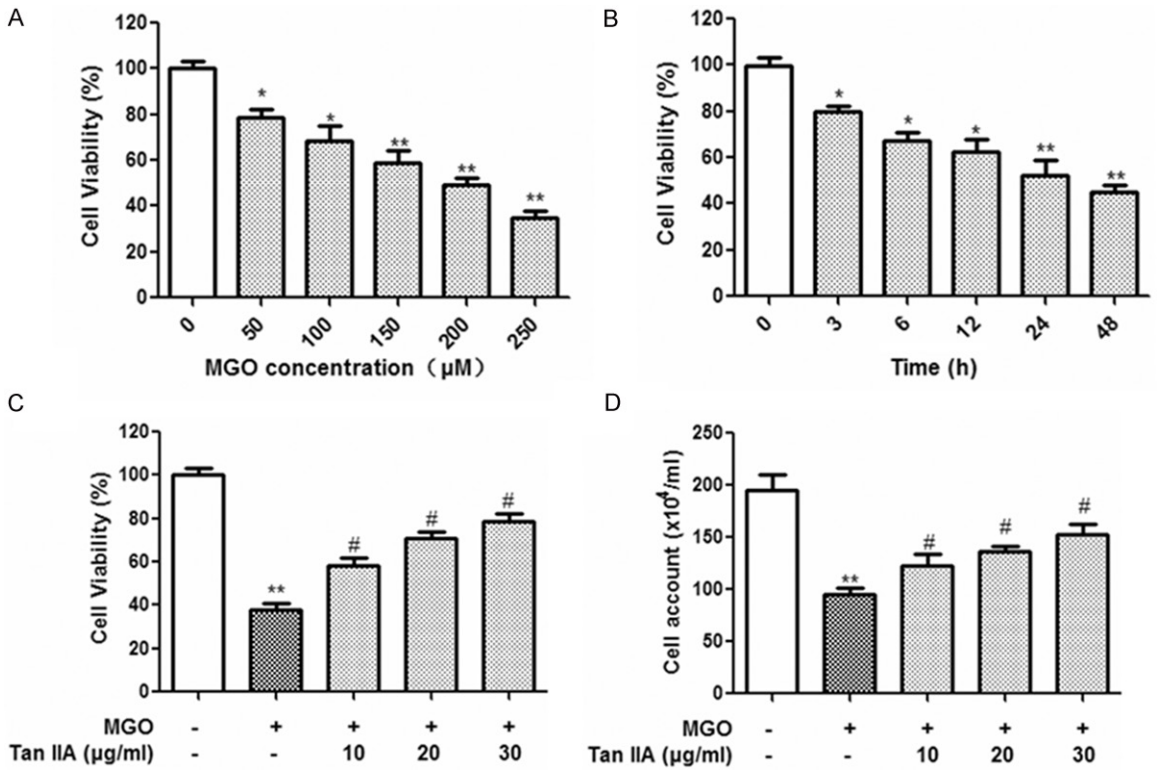

Figure 1. Effect of methylglyoxal (MGO) and tanshinone IIA on cell viability. A. HBMEC were incubated with different concentration of MGO (0, 50, 100, 150, 200 and 250 μM) for 24 h. B. HBMEC were incubated with 200 μM MGO for different time points (0, 3, 6, 12, 24 and 48 h). C. HBMEC were incubated with Tan IIA (10 μg/ml, 20 μg/ml and 30 μg/ml) prior to 200 μM MGO exposure. After 24 h MGO treatment, cell viability was determined by MTT assay. D. Cell counts were assessed by trypan blue exclusion test. **P*<0.05, ***P*<0.01 vs. control group; #*P*<0.05 vs. MGO-treated group.

were presented as means ± SD and treated for statistics analysis by SPSS program. Comparison between groups was made using ANOVA and statistically significant difference was defined as $P<0.05$.

Results

Effect of MGO and tanshinone IIA on cell viability

First of all, the effect of MGO in the cultured HBMEC was determined by MTT assay. As shown in the figures, the viability of cells subjected to MGO showed a concentration-dependent decrease when exposed to MGO at different concentrations from 50 to 250 μM (**Figure 1A**). Then, we incubated cells with 200 μM MGO at different time points (0, 3, 6, 12, 24 and 48 h). Results showed that MGO treatment induced a time-dependent reduction in the cell viability (**Figure 1B**). These data demonstrated that MGO induced cell injury in a dose-and time-dependent manner. Next we incubated HBMEC with different concentrations of Tan IIA for 24 h and found no significant differences between control group and Tan IIA-treated groups (data not shown). Then cells were treated with Tan IIA (10 μg/mL, 20 μg/mL and 30 μg/mL) prior to MGO (200 μM) exposure. We found that Tan IIA pretreatment significantly increased the cell viability in a dose-dependent manner (**Figure 1C**). As measured by cell account, similar protective effect of Tan IIA was also observed (**Figure 1D**).

Tanshinone IIA attenuated MGO-induced AGEs formation and oxidative stress in HBMEC

Accumulation AGEs and generation of ROS have been involved in pathogenesis of diabetic complications. We found that Tan IIA pretreatment dose-dependently decreased AGEs accumulation compared to MGO-treated group (**Figure 2A** and **2B**). Moreover, the production of ROS (**Figure 2C**), TBARS (**Figure 2D** and H_2O_2

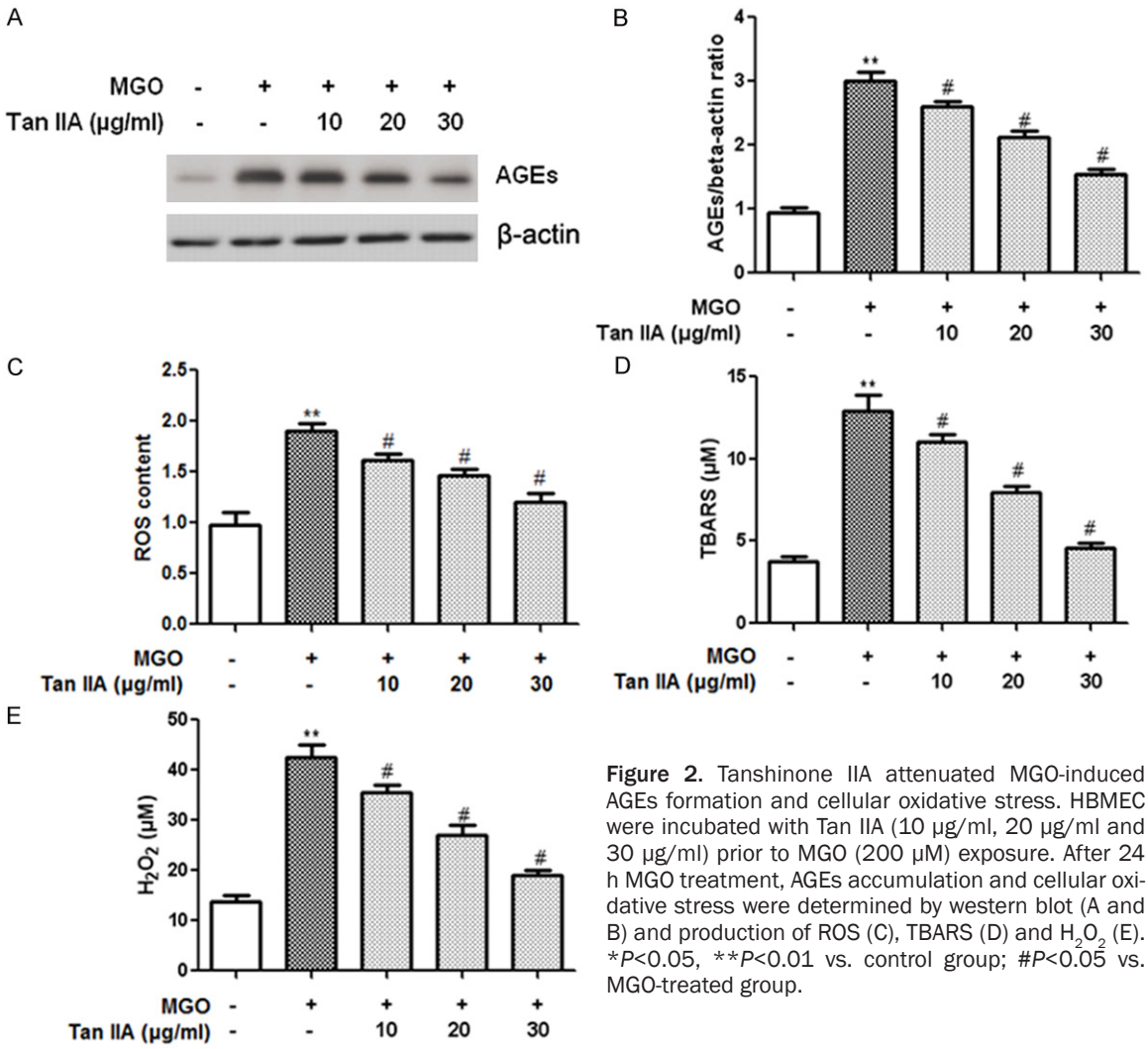

Figure 2. Tanshinone IIA attenuated MGO-induced AGEs formation and cellular oxidative stress. HBMEC were incubated with Tan IIA (10 μg/ml, 20 μg/ml and 30 μg/ml) prior to MGO (200 μM) exposure. After 24 h MGO treatment, AGEs accumulation and cellular oxidative stress were determined by western blot (A and B) and production of ROS (C), TBARS (D) and H_2O_2 (E). **P*<0.05, ***P*<0.01 vs. control group; #*P*<0.05 vs. MGO-treated group.

(**Figure 2E**) was remarkably enhanced after MGO treatment, which was inhibited by pretreatment with Tan IIA. Collectively, these data suggested that Tan IIA protected against MGO-induced cell injury in HBMEC by reducing AGEs accumulation and oxidative stress.

Tanshinone IIA treatment reduced cell apoptosis induced by MGO

We next investigated the effect of Tan IIA on cell apoptosis by flow cytometry. AnnexinV/PI staining showed that MGO treatment obviously increased cell apoptosis compared to the control group. However, pretreatment with Tan IIA significantly decreased cell apoptosis compared to MGO-treated group (**Figure 3**). In addition, the expression levels of apoptosis-related protein were assessed by western blot analysis. As shown in the figures, MGO exposure increased the levels of p53, Bax and cyto C, and decreased Bcl-2 expression. However, such molecular changes were reversed by Tan IIA treatment (**Figure 4A** and **4B**). Together, these data indicated that Tan IIA could decrease MGO-induced cell apoptosis in HBMEC.

Tanshinone IIA protects MGO-induced cell injury through inhibiting MAPK activation

Mitogen activated protein (MAP) kinase family has been associated with cell proliferation and apoptosis. To further characterize the molecular mechanisms involved in the protective effect of Tan IIA, we analyzed the MAP kinases including JNK, ERK1/2, and p38 by western blot. Results showed that pretreatment with

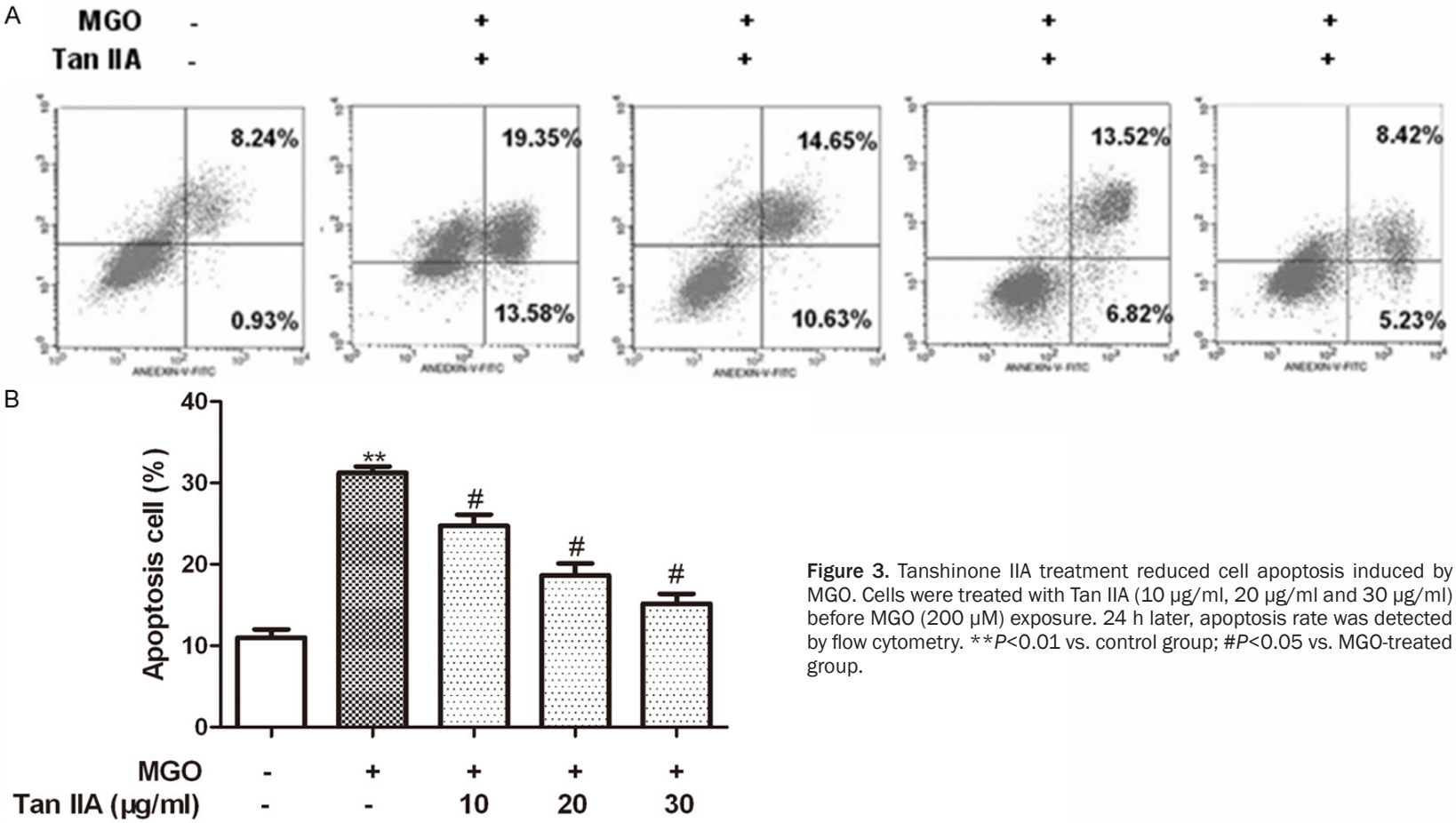

Figure 3. Tanshinone IIA treatment reduced cell apoptosis induced by MGO. Cells were treated with Tan IIA (10 μg/ml, 20 μg/ml and 30 μg/ml) before MGO (200 μM) exposure. 24 h later, apoptosis rate was detected by flow cytometry. $**P<0.01$ vs. control group; $\#P<0.05$ vs. MGO-treated group.

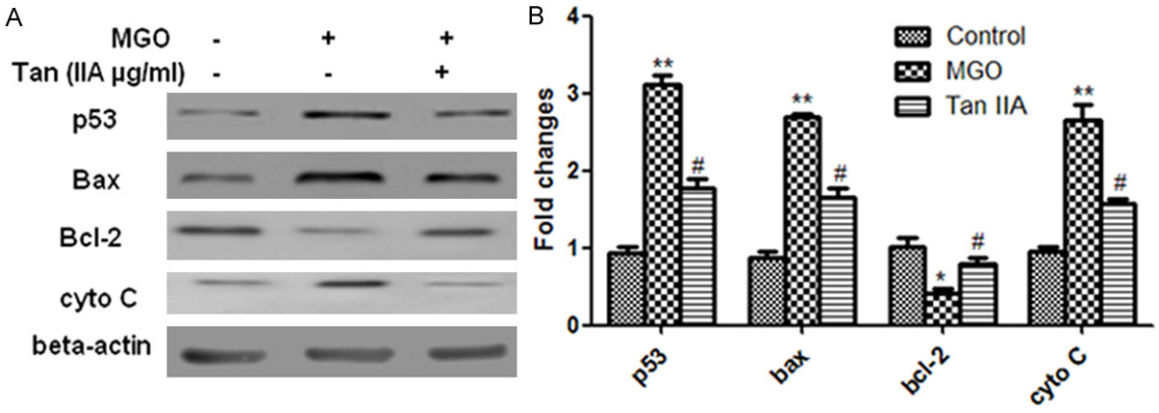

Figure 4. Tanshinone IIA modulated the expression of apoptosis-related proteins. Cell lysates were harvested and subjected to western blot analysis (A). Expression levels of apoptosis-related proteins (p53, bax and bcl-2 and cyto C) were measured by western blot (B). **P*<0.05, ***P*<0.01 vs. control group; #*P*<0.05 vs. MGO-treated group.

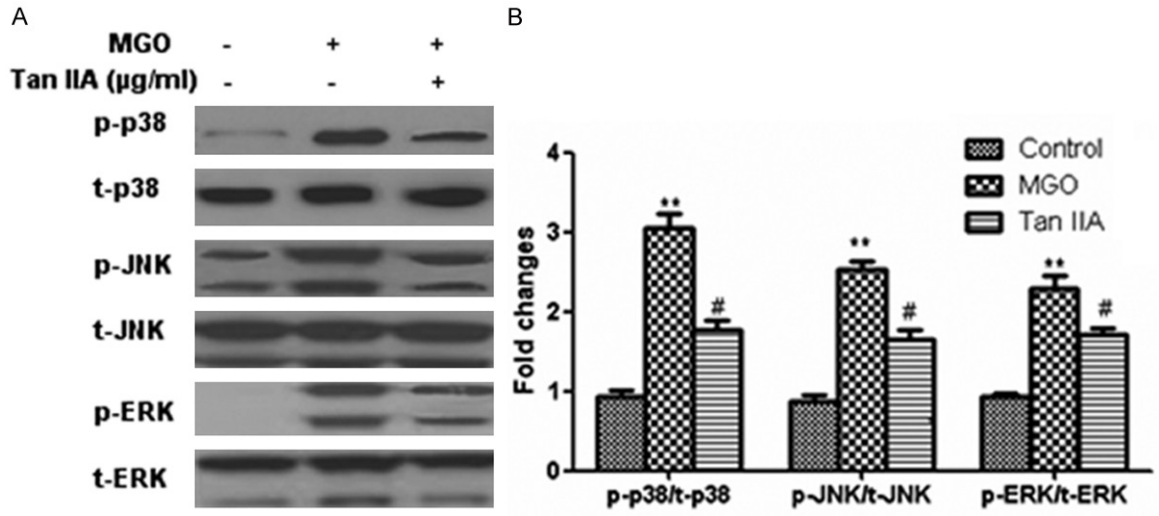

Figure 5. Tan IIA regulated the activity of MAPK. HBMEC were treated with Tan IIA (30 μg/ml) before MGO (200 μM) exposure. 24 h later, western blot was performed to determine the phosphorylation levels of p38, JNK and ERK (A). Relative band intensities were used for quantification of p-38, p-JNK and p-ERK (B). **P*<0.05, ***P*<0.01 vs. control group; #*P*<0.05 vs. MGO-treated group.

Tan IIA significantly reduced the phosphorylation levels of p38, JNK and ERK compared to the MGO-treated group (**Figure 5A** and **5B**). These results indicated that Tan IIA protected MGO-induced cell injury partially through inhibiting MAPK activation in HBMEC.

Discussion

Tan IIA is one of the major diterpenes from SM and has been shown to possess a protective effect on the endothelial cells [15, 16]. The present study investigated the biological effect of Tan IIA on HBMEC, and for the first time, we found that Tan IIA treatment produced a protective effect on methylglyoxal-induced injury in the cultured HBMEC.

It is well known that patients with either type 1 or type 2 diabetes have high serum concentration of MGO, suggesting that MGO may be implicated in vascular complications of diabetes [17]. Under hyperglycemic conditions, Maillard reaction occurs in which glucose and fructose-precursors of triose phosphate promote MGO formation by nonenzymatic elimination of phosphate [18, 19]. In our study, MGO treatment caused endothelial cells injury in a concentration-and time-dependent manner.

However, pretreatment Tan IIA reversed the cell injury induced by MGO in the cultured HBMEC.

In the development of diabetic complications, MGO can modify cellular proteins to form cross-links of amino groups, and then generates AGEs [20]. Numerous studies have highlighted the causal relationship between MGO-derived AGEs and diabetic complications as well as effects of AGEs on endothelial dysfunction [21, 22]. It was demonstrated that AGEs could induce apoptosis of corneal endothelial cells by increasing cellular oxidative stress [23]. Our data showed that AGEs accumulation was significantly increased after 24 h MGO treatment, which was attenuated by Tan IIA treatment. Moreover, Tan IIA treatment also alleviated the production of ROS, TBARS and H_2O_2 in the cultured HBMEC. These results suggested that Tan IIA protected HBMEC by inhibition of AGEs accumulation and oxidative stress.

Endothelial cell apoptosis, the process of programmed cell death, is observed in multicellular organisms and plays an important role in the process of diabetes-associated vascular complications [24]. In our study, Tan IIA pretreatment significantly attenuated apoptosis induced by MGO exposure in HBMEC. We further investigated the expression changes of apoptosis-related proteins. Not surprisingly, western blot analysis showed that pretreatment with Tan IIA attenuated the expression of p53, bax and cyto C, and enhanced the bcl-2 expression. Together, these data demonstrated that Tan IIA treatment inhibited MGO-induced cell apoptosis in the cultured HBMEC.

MAPK family has been shown to be involved in many physiological and pathological processes [25]. Accumulating studies have shown that the sustained activation of MAPK signaling pathway plays an important role in the regulation of cell proliferation, pro-apoptotic proteins expression and ROS production [26, 27]. We found that Tan IIA treatment could decrease the phosphorylation levels of p38, JNK and ERK, indicating that Tan IIA protected MGO-induced cell injury through inhibiting MAPK activation.

In summary, we demonstrated that tanshinone IIA pretreatment protected against MGO-induced injury in the cultured HBMEC through modulating MAPK activity. Our study provides significant experimental data for developing tanshinone IIA as therapeutic agents for prevention and treatment of vascular complications associated with diabetes.

Acknowledgements

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Disclosure of conflict of interest

None.

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