Original Paper

Protective Effects of Oxymatrine on Vascular Endothelial Cells from High-Glucose-Induced Cytotoxicity by Inhibiting the Expression of A_{2B} Receptor

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Key Words

A_{2B} receptor • Oxymatrine • Human umbilical vein • Endothelial cells • Diabetes mellitus

Abstract

Background/Aims: Diabetes mellitus (DM) has become an increasingly epidemic metabolic disease. Vascular endothelial cells play a key role in developing the cardiovascular complications of DM. The A_{2B} receptor is expressed in vascular endothelial cells, and may help regulate the function of endothelial cells. The aim of this study was to investigate the protective effects of oxymatrine (OMT) on human umbilical vein endothelial cells (HUVECs) from high glucose-induced cytotoxicity. **Methods:** Homology modeling and molecular docking analysis were used to detect the binding sites between the adenosine A_{2B} receptor and OMT. HUVECs were cultured with control (5.5 mM) or elevated glucose (22.2 mM) in the presence or absence of 3 μM OMT or A_{2B} siRNA for 3 days. The MTS cell viability assay was used to measure the toxicity of high glucose on HUVECs and the protective effect of OMT or A_{2B} siRNA. The expression of the adenosine A_{2B} receptor and CCL5 in HUVECs was detected with real-time quantitative PCR (qPCR) and Western blotting methods in each group. Levels of IL-1β and TNF-α were measured using an enzyme-linked immunosorbent assay (ELISA) kit, and the concentration of NO was detected with the nitrate reductase method. Monocyte chemotactic activity in each group was detected using Transwell chambers. Furthermore, the phosphorylation of p38 and ERK1/2 in each group was observed through the Western blotting method. **Results:** Homology modeling and molecular docking analysis showed that OMT contains well-fitted

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binding sites to the A_{2B} receptor. After chronic culture at high glucose, the rate of cell viability was significantly lower than that of the control group. After co-treatment with OMT or A_{2B} siRNA, cell viability was significantly increased compared with the high-glucose group. The results from real-time quantitative RT-PCR (qRT-PCR) and Western blotting indicated that high glucose could increase the expression of A_{2B} receptors in HUVECs, an effect that was inhibited by OMT. In addition, the results revealed that the expression of CCL5, IL-1 β and TNF- α was increased in the high-glucose group, and that the NO produced by HUVECs decreased due to hyperglycemia; however, co-culture with OMT or A_{2B} siRNA abolished these effects. Meanwhile, the chemotaxis activity of monocytes to HUVECs cultured in high-glucose medium was enhanced 2.59-fold compared to the control cells. However, the inflammatory reactions in HUVECs were completely relieved by co-treatment with OMT or A_{2B} siRNA. Moreover, the phosphorylation of p38 and ERK1/2 in HUVECs in the high-glucose group was significantly higher than that of the control group; these effects were reversed after co-treatment with OMT or A_{2B} siRNA. **Conclusion:** OMT may protect the HUVECs from high glucose-induced cytotoxicity through inhibiting the expression of A_{2B} receptor and inflammatory factors as well as decreasing the phosphorylation of p38 and ERK1/2.

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Introduction

Diabetes mellitus (DM) is caused by an absolute or a relative deficiency of insulin secretion, which results in hyperglycemia and dyslipidemia. Vascular endothelial cells play a key role in the development of cardiovascular complications of diabetes [1]. They not only form a barrier that separates the inner and outer blood vessels but also act as an important organ that has the function of regulating vascular functions. Diabetic vascular endothelial dysfunction is considered to be the earliest manifestation of disease that occurs before anatomic evidence of macrovascular diseases appears [2]. Endothelial dysfunction has become an important initiating and key factor in the development of diabetic vascular complications.

The mechanism of the toxic effect of hyperglycemia or high levels of fatty acids on vascular endothelial cells remains to be fully elucidated. Using endothelial cells cultured *in vitro* makes it easier to observe the effects of hyperglycemia and hyperlipidemia on cell injury [3]. Cell culture has fewer interfering factors, and the results of experiments may be more meaningful [4].

Adenosine is an important bioactive substance in the human body. Adenosine is not only involved in cellular enzyme reactions but also acts as an active ligand in regulating many cell functions [5, 6]. It has been shown that intracellular adenosine exerts its physiological role only after it reaches the extracellular adenosine receptor by diffusion or active transport. Adenosine receptors are divided into 4 subtypes: A₁, A_{2A}, A_{2B} and A₃, depending on the ability of the receptor to bind adenosine and whether its activity regulates adenosine cyclase [7, 8].

It has been found that the A_{2B} receptor is expressed in vascular endothelial cells, and may regulate the function of endothelial cells [9]. White blood cells seep into endothelial cells to impair their barrier function, causing fluid loss and edema. In the late stage of inflammation during the formation of new blood vessels, A_{2B} receptor activation can stimulate human microvascular endothelial cells to release VEGF and fibroblast growth factor, resulting in increased endothelial cell proliferation, chemotaxis and capillary formation [10]. A_{2B} receptor may promote the expression and release of a variety of pro-inflammatory mediators (e.g. IL-4, IL-8, IL-6, IL-13, IL-19 and monocyte chemoattractant protein) in the pathological process of chronic lung diseases [11]. Figler et al. reported that the expression of A_{2B} receptor in vascular endothelial cells was increased in diabetes, leading to an increase in the secretion of inflammatory factor IL-6 [12]. At present, not much is known about the function of A_{2B} receptor and its implication in diabetic vascular endothelial dysfunction.

Oxymatrine (OMT) is an alkaloid with a four-ring quinazoline structure extracted from *Sophora flavescens* Ait. OMT has shown anti-inflammatory, antitumor, antiviral, and anti-

arrhythmic effects [13, 14]. This study aimed to investigate the potential protective effect of OMT on human vascular endothelial cells against high-glucose toxicity and the underlying mechanism, with the primary goal of looking for a new target for the prevention and treatment of diabetic complications caused by vascular endothelial dysfunction.

Materials and Methods

Cell culture and treatment

HUVECs (ATCC PCS-100-010) were cultured at 37°C and 5% CO₂ in RPMI 1640 (Invitrogen; Gibco, Germany), containing 10% FBS, 100 U/ml penicillin G, and 100 mg/ml streptomycin. For experiments, HUVECs were treated with high glucose to mimic diabetic conditions for 3 days. There were 6 groups in the study according to the different treatments for the cells: control glucose (5.5 mM) (control group); high glucose (22.2 mM group); high glucose (22.2 mM) + scramble siRNA (22.2 mM + scramble siRNA group); control glucose (5.5 mM) + 3 μM OMT (control + OMT group); high glucose + 3 μM OMT (22.2 mM + OMT group); high glucose (22.2 mM) + A_{2B} siRNA (22.2 mM + A_{2B} siRNA group).

Cell viability

An MTS assay was performed to determine cell viability and proliferation according to the manufacturer's protocol (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega Company) as previously described [3]. Briefly, HUVECs were seeded in 96-well plates with MTS solution for 2 h. The optical density of formazan production in each well was read at 490 nm with a microplate reader (Rayto, RT-600 Microplate Reader, Shenzhen, China). For each experiment, eight individual wells of each treatment condition were tested. Each experiment was repeated three times.

Homology modeling and molecular docking analysis to detect the binding site of OMT to the A_{2B} receptor

Molecular docking is a computer simulation tool that attempts to predict the binding mode of a ligand in the active site of a protein. Molecular docking studies mimic the spontaneous interaction of a ligand with the protein. Molecular docking computations were performed using AutoDock vina [15]. The technique of docking is to position the ligand in different orientations and conformations within the binding site to calculate optimal binding geometries and energies. Therefore, after the docking procedure the proper conformation of the ligand in the active site of the protein is obtained and used for the calculation of molecular descriptors. For each ligand, a number of configurations called poses are generated and scored [16]. A_{2B} receptor was selected as the docking receptor. Homology modeling (or comparative modeling) relies on evolutionarily related structures (templates) to generate a structural model of a protein of interest (target). Through online tools of Swiss-model [17] <https://swissmodel.expasy.org/>, we obtained the three-dimensional structure of A_{2B} receptor. Input files of amino acid (A_{2B} receptor, NP_000667, Refseq protein number) and a ligand (OMT) were prepared with AutoDock Tools (ADT) [16] and Python scripts named `prepare_ligand4.py` and `prepare_receptor4.py`, which are associated with the AutoDock4.2 program. The Pubchem CID of the ligand (OMT) was 114850; the CAS number was 16837-52-8. The binding pocket position in target protein was specified with the ADT molecular viewer. The parameters were kept at their default values. Finally, the output files were viewed using PyMol 2.0 [18].

A_{2B} receptor small interference RNA treatment

The small interfering RNAs (siRNAs) specific for the A_{2B} receptor were purchased from RIBOBIO CO. (Guangzhou, China). siRNA oligonucleotides targeted specifically to A_{2B} receptor were used in this experiment [19]. There were three siRNA duplexes targeting different encoding regions of the A_{2B} receptor mRNA, A_{2B} siRNA1, A_{2B} siRNA2 and A_{2B} siRNA3, that were designed and synthesized. These siRNAs were transiently transfected into the HUVECs. Western blotting and qRT-PCR were used to confirm the efficacy of A_{2B} siRNA for suppression of A_{2B} receptor protein and mRNA. The A_{2B} siRNAs were transfected into cultured cells by Lipofectamine2000 (Invitrogen). In detail, the A_{2B} siRNAs and Lipofectamine2000 were diluted in Opti-MEM® medium without serum, and the final concentration of A_{2B} siRNAs was 50 nM. The optimal A_{2B} siRNA was selected based on qRT-PCR and Western blotting analysis.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

QRT-PCR was performed using specific primers (Sangon, Beijing, China) on an Applied Biosystems 7500 system (ABI, Waltham, American). The annealing and melting temperatures were optimized for each primer set. GAPDH was amplified to ensure cDNA integrity and to normalize expression levels. Each real-time PCR was performed in duplicates. All experiments were repeated at least three times. The oligonucleotides used for amplification were as follows: for A_{2B} receptor, sense 5'-ACCGAGACTTCCGCTACACTT-3' and antisense 5'-CTGACCATTCCTCCACTCTTGAC-3'; for CCL5, sense 5'-CCTGCTGCTTTGCCTACATT-3' and antisense 5'-CCAGACTTGCTGTCCCTCTC-3'; and for GAPDH, sense 5'-CAGGAGGCATTGCTGATGAT-3' and antisense 5'-GAAGGCTGGGGCTCATT-3'. The PCR products were amplified using the following cycling parameters: 94°C for 5 min; followed by 35 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s; and, finally, a single cycle at 72°C for 10 min.

Western blotting

Following treatment under different culture conditions, HUVECs were harvested in lysis buffer (RIPA:PMSF:All-in-One; 100:1:1, Solarbio, China) and incubated for 30 min on ice. Protein concentrations were determined using a plate reader (Rayto, RT-600 Microplate Reader, Shenzhen, China). The same quantity of protein per lane was resolved on 12% SDS-PAGE gels, and then transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% milk in TBS-T (0.1 M Tris-HCl, pH 7.4, 0.9% NaCl, 0.1% Tween-20) and then incubated with primary rabbit anti-antibodies against β -actin (ZSbio, Beijing, China), the phosphorylated or total forms of ERK1/2 and p38 MAPK (Cell Signaling Technology Inc, Boston, MA, USA), CCL5 (Abcam Inc., Shanghai, China) or A_{2B} receptor (Fisher Scientific Inc, Shanghai, China). Subsequently, the membranes were incubated with horseradish peroxidase-conjugated secondary goat anti-rabbit IgG (Sigma, MO, USA). The membranes were washed and quantitated using an enhanced chemiluminescence detection system (ECL; Amersham, Buckinghamshire, UK). The band densities were normalized against the β -actin internal control and analyzed by Image-J system software.

Determination of NO production

After HUVECs were cultured for 3 days with different treatments in 24-well culture plates, each group was set up with three wells per compound. Then, the culture solution was collected to measure the concentrations of nitric oxide (NO) by a kit supplied by Nanjing Jiancheng Bioengineering Institute. The nitrate reductase method was used in the kit to turn NO-derived nitrate into nitrite, thus, the nitrite concentrations in the culture media were assayed. The absorbance of nitrite was measured by a microplate reader (Rayto RT-6000, Ruixin) at 550nm.

The calculation formula of the concentration of NO is as follows: concentration of NO ($\mu\text{mol/L}$) = [(absorbance of treated wells – absorbance of blank wells) / (absorbance of standard wells – absorbance of blank wells) $\times$ standard concentration (100 $\mu\text{mol/L}$)] $\times$ dilution times of the sample before measuring.

Chemotaxis assay

A monocyte chemotactic activity assay was performed in 12-well Transwell chambers fitted with 5- μm pore polycarbonate membranes (Corning Inc., USA). HUVECs were cultured in the lower chamber in the designated conditions. U937 cells loaded with BCECF-AM were added to the upper chamber at 4×10^6 cells/ml and co-cultured with HUVECs in the lower chamber at 37°C for 2 h. Cells remaining in the top chamber of the filter were aspirated and the surface was washed twice with RPMI 1640 medium. The plate was then centrifuged (312 $\times$ g, 5 min) and the filter was removed. The cells were observed by fluorescence microscopy (AMG EVOS/fl). The quantitation of fluorescent cells was carried out using a fluorescence reader (Tecan infinite M200, Switzerland).

ELISA measurement of cytokine levels in the culture supernatant

The culture supernatants of cells were collected after cultivation in high glucose with different treatments for 3 days. Samples were maintained at room temperature for 30 min and centrifuged at 3000 rpm for 10 min. The culture supernatants were collected and stored at -20°C. Levels of IL-1 β and TNF- α were quantified using an enzyme-linked immunosorbent assay (ELISA) kit following the manufacturer's protocol (Senxiong Co., Shanghai, China). The reactions were read using an ELISA reader (Rayto, RT-6000, USA) at 450 nm.

Statistical analysis

All results are expressed as the mean ± SEM. Differences between treatment groups were analysed by Student's t-test or, where appropriate, ANOVA followed by Dunnett's post hoc test for multiple comparisons. A P-value of < 0.05 was considered statistically significant.

Results

Homology modeling and molecular docking analysis discloses the binding site of OMT to the A_{2B} receptor

Molecular docking of OMT on the A_{2B} receptor was visualized by AutoDock 4.2. The steps were as follows. Protein molecules were dehydrated and hydrogenated. After calculation of the Gibbs free energy, the pdbqt file of the A_{2B} receptor was produced by the Grid function of the ADT Tools toolbar. For the ligand (OMT), the parameter was set by the ADT Tools column, and the pdbqt file of OMT was saved. The docking score of the A_{2B} receptor and OMT (kcal/mol) showed that OMT had a perfect fit to interact with the A_{2B} receptor protein (see Table 1). The perfect match enabled the OMT to interact with the A_{2B} receptor (see Fig. 1).

Efficacy of A_{2B} siRNA in the interfering with the A_{2B} receptor in HUVECs

There were three siRNA duplexes targeting different encoding regions of A_{2B} receptor mRNA, named A_{2B} siRNA1, A_{2B} siRNA2 and A_{2B} siRNA3, that were designed and synthesized. These siRNAs were transiently transfected into the HUVECs. Western blotting and qRT-PCR were used to confirm the efficacy of A_{2B} siRNA for suppression of A_{2B} receptor protein and mRNA. The results showed that transfection of the A_{2B} siRNA2 resulted in a significant decrease in A_{2B} protein and mRNA expression (see Fig. 2). Thus A_{2B} siRNA2 that targeted sequence 5'- GAAAGCTGCTGCCTTGTGA -3' was selected for further experiments.

Protective effects of OMT or A_{2B} siRNA on HUVECs from high glucose induced cytotoxicity

Exposure to high glucose significantly reduced the viability of HUVECs significantly, and co-culture with OMT or A_{2B} siRNA abolished the cytotoxicity effect of high glucose (Fig. 3).

Effects of OMT on A_{2B} receptor expression in HUVECs cultured in high glucose

The expression of the A_{2B} receptor was measured by qRT-PCR. The results showed that in the 22.2 mM group, A_{2B} receptor mRNA was significantly higher than that in the control

Table 1. MOE score of A_{2B} docking and oxymatrine (kcal/mol). Explanation: The predicted binding affinity is in kcal/mol (energy). *rmsd: RMSD values are calculated relative to the best mode and use only movable heavy atoms. Two variants of RMSD metrics are provided, rmsd/lb (RMSD lower bound: matches each atom in one conformation with itself in the other conformation, ignoring any symmetry) and rmsd/ub (RMSD upper bound: rmsd/lb(c1, c2) = max(rmsd'(c1, c2), rmsd'(c2, c1)), and rmsd' matches each atom in one conformation with the closest atom of the same element type in the other conformation), differing in how the atoms are matched in the distance calculation. There is a strong reaction between ligand and protein when the binding affinity is higher than -6.0 kcal/mol. The rmsd/lb and rmsd/ub of models 1 to 5 are much the same, as well as of models 6-8, which indicates that those models are locating in one docking pocket. In sum, the molecular docking of oxymatrine on a model-h protein A_{2B} is stable

Mode	Affinity (kcal/mol)	Dist From Best Mode	
		RMSD lb.	RMSD u.b.
1	-7.0	0	0
2	-7.0	30.656	32.304
3	-6.8	19.437	21.561
4	-6.7	1.896	3.548
5	-6.6	1.937	5.525
6	-6.6	36.896	38.197
7	-6.5	31.908	33.619
8	-6.3	1.853	4.329
9	-6.0	28.247	30.096

Fig. 1. Molecular docking of OMT on the A_{2B} receptor. Simulation modeling of OMT docking with the A_{2B} receptor protein was performed by a computer. Molecular docking of OMT on the A_{2B} receptor was generated by AutoDock 4.2 (Fig. A). Enlarged view indicated that the perfect match enabled the OMT to interact with the A_{2B} receptor (Fig. B, C, D).

Fig. 2. The effect of different siRNAs on the expression of A_{2B} receptor mRNA and protein. Huvecs were seeded into 6-well plates and transfected with three siRNAs targeting the A_{2B} receptor (siRNA1, siRNA2, and siRNA3) or scramble siRNA. Untransfected cells served as a control. A_{2B} receptor protein and mRNA levels were detected by Western blotting and qRT-PCR. Expression levels of A_{2B} receptor are presented as mean $\pm$ SD. Triplicate experiments were performed with almost identical results. ** $p < 0.01$ vs. control.

group ($p < 0.01$). The results also showed that the expression of the A_{2B} receptor in the 22.2 mM + OMT group was significantly lower than that in the 22.2 mM group ($p < 0.01$), and the A_{2B} receptor mRNA in the 22.2 mM + A_{2B} siRNA group was significantly lower than that in the 22.2 mM + scramble siRNA group ($p < 0.01$) (Fig. 4).

Furthermore, the A_{2B} receptor at the protein level was measured by Western blotting. The results showed that the expression of the A_{2B} receptor protein in the 22.2 mM group was significantly higher than that in the control group ($p < 0.01$); the expression of the A_{2B} receptor protein in the 22.2 mM + A_{2B} siRNA group was significantly lower than that in the 22.2 mM + scramble siRNA group ($p < 0.01$); the expression of the A_{2B} receptor protein in the

Fig. 3. Viability of HUVECs in high glucose media with or without OMT or A_{2B} siRNA. MTS assays were performed after HUVECs were cultured with control (5.5 mM) or elevated glucose (22.2 mM) in the presence or absence of 3 μ M OMT or A_{2B} siRNA for 3 days. Values are the mean $\pm$ SEM of 8 observations. ** $p < 0.01$ vs. control.

Fig. 4. Effects of OMT or A_{2B} siRNA on expression of the A_{2B} receptor mRNA in HUVECs cultured in high glucose media. A_{2B} expression at the mRNA level was detected by qRT-PCR (normalized to individual GAPDH internal control). Values are the mean $\pm$ SEM of 6 observations. ** $p < 0.01$ vs 22.2 mM group or 22.2mM + scramble siRNA.

22.2 mM + OMT group was significantly lower than that in the 22.2 mM group ($p < 0.01$) (Fig. 5).

Effects of OMT or A_{2B} siRNA on the CCL5 expression of HUVECs and chemotaxis activity of monocytes to HUVECs at high glucose

The CCL5 expression in HUVECs was detected by qRT-PCR. It was found that CCL5 mRNA in the 22.2 mM group was significantly higher than that in the control group ($p < 0.01$). The expression of CCL5 in the 22.2 mM + OMT group was significantly lower than that in the 22.2 mM group ($p < 0.05$), and the expression of CCL5 mRNA in the 22.2 mM + A_{2B} siRNA group was significantly lower than that in the 22.2 mM + scramble siRNA group (Fig. 6).

The chemotaxis activity of monocytes to HUVECs cultured in high-glucose medium was enhanced 2.59-fold compared with the control cells (Fig. 7). The inflammatory chemotaxis reactions in HUVECs were completely relieved by co-treatment with 3 μ M OMT or A_{2B} siRNA (Fig. 7).

Effects of OMT or A_{2B} siRNA on NO production by HUVECs

The NO production by HUVECs in the high-glucose group was significantly lower than in the control group. The glucose suppressed NO production was increased significantly by co-treatment with 3 μ M OMT or A_{2B} siRNA (Fig. 8).

Effects of OMT or A_{2B} siRNA on cytokine levels in the culture supernatant

The culture supernatants of cells were collected after cultivation in high glucose with different treatments for 3 days. Levels of IL-1 β and TNF- α were measured using ELISA kits. The results showed that IL-1 β and TNF- α levels in the 22.2 mM group were significantly higher than in the control group. The expression levels of IL-1 β and TNF- α in the 22.2 mM + OMT group were significantly lower than those in the 22.2 mM group ($p < 0.05$), and the expression levels of IL-1 β and TNF- α in the 22.2 mM + A_{2B} siRNA group were significantly lower than in the 22.2 mM + scramble siRNA group ($p < 0.01$) (Fig. 9).

Fig. 5. Effect of OMT or A_{2B} siRNA on expression of the A_{2B} receptor protein in HUVECs cultured in glucose medium. A_{2B} receptor expression at the protein level was assessed by Western blotting. The stain values (integrated optical density) of A_{2B} receptor protein expression (normalized to each β-actin internal control) were obtained using image analysis. Values are the mean ± SEM of 6 observations. **p<0.01 vs 22.2 mM group or 22.2mM + scramble siRNA.

Fig. 6. Effects of OMT or A_{2B} siRNA on expression of CCL5 mRNA in HUVECs cultured in high glucose. CCL5 expression at mRNA level was detected by qRT-PCR (normalized to individual GAPDH internal control). Values are the mean ± SEM of 6 observations. **p<0.01 vs 22.2 mM group or 22.2mM + scramble siRNA.

Effects of OMT A_{2B} siRNA on the phosphorylation of ERK1/2 and p38 in HUVECs cultured in high glucose medium

There was no significant change in total ERK1/2 MAPK protein among the 6 groups of HUVECs. In the 22.2 mM group, the level of P-ERK1/2 was significantly higher than that in the control group (p<0.01). P-ERK1/2 in the 22.2 mM+OMT group was significantly lower than that in the 22.2 mM group (p<0.01) and similarly the level of P-ERK1/2 in the 22.2 mM + A_{2B} siRNA group was significantly lower than that in the 22.2 mM + scramble siRNA group (p<0.01) (Fig. 10).

There was also no significant change in total p38 MAPK protein expression in any group of HUVECs. In the 22.2 mM group, the phosphorylation of p38 was significantly higher than that in the control group (p<0.01). The phosphorylation of p38 in the 22.2 mM+OMT group

Fig. 7. Effects of OMT or A_{2B} siRNA on increased chemotaxis of HUVECs to monocytes due to high glucose. HUVECs and BCECF-labeled U937 cells were cultured in 2-chambered trans-well plates with control (5.5 mM) or elevated glucose (22.2mM) in the presence or absence of 3 μ M OMT or A_{2B} siRNA for 3 days. After washing and centrifugation, the plates were observed under fluorescence microscopy. Fluorescent cells in each well were counted and the data in the bar graph are expressed as the fold over control. Values are the mean $\pm$ SEM of 6 observations. ** p < 0.01 vs 22.2 mM group or 22.2mM + scramble siRNA. Scale bars, 500 μ m.

was significantly lower than that in the 22.2 mM group ($p < 0.01$), and the phosphorylation of p38 in the 22.2 mM + A_{2B} siRNA group was significantly lower than that in the 22.2 mM + scramble siRNA group ($p < 0.01$) (Fig. 11).

Discussion

Vascular endothelial cells play a key role in regulating the function of blood vessels. Vascular lesions directly affect the prognosis and survival of patients with DM [20, 21]. Vascular endothelial cells are involved in inflammation and immune response by secreting a series of bioactive substances, such as prostacyclin, NO, endothelin and von Willebrand factor [22]. In addition to the formation of NO which promotes vasodilation, endothelial cells also modulate the migration of leukocytes through releasing inflammatory chemokines, cytokines, and cell surface adhesion molecules [23]. Inflammation, trauma and other stress

Fig. 8. Effects of OMT or A_{2B} siRNA on NO products of HUVECs. The NO products of HUVECs were measured after cultivation in high glucose with different treatments for 3 days. Values are the mean ± SEM of 6 observations. **p<0.01 vs Control group; ##p<0.01 vs 22.2 mM group or 22.2mM + scramble siRNA.

Fig. 9. Effects of OMT or A_{2B} siRNA on cytokine levels in culture supernatants. The culture supernatants of cells were collected after cultivation in high glucose with different treatments for 3 days. Levels of IL-1β and TNF-α were measured using ELISA kits. Values are the mean ± SEM of 6 observations. **p<0.01 vs control group; **p<0.01 vs 22.2 mM group or 22.2mM + scramble siRNA.

injuries stimulate endothelial cells to increase vascular permeability and adhesion molecule expression, leading to edema, adhesion of leukocytes, and a subsequent inflammatory cascade reaction [24]. At high blood glucose, vascular endothelial cells undergo pathological changes, and their secretory activity changes.

The A_{2B} receptor plays an important role in regulating the function of vascular endothelial cells. The A_{2B} receptor is expressed in vascular endothelial cells and may be involved in the regulation of pro-inflammatory responses in vascular endothelial cells [25]. Studies in A_{2B} receptor knockout mice showed inhibition of the infiltration of neutrophils into tissues under hypoxic conditions [26, 27]. Pro-inflammatory effects of A_{2B} receptors have been seen in murine lung inflammation models and in patients with asthma or chronic obstructive pulmonary disease, suggesting the potential therapeutic value of targeting A_{2B} receptors as an anti-inflammation measure to prevent the progression of these diseases [28, 29]. It has been reported that the A_{2B} receptor may promote the expression and release of the inflammatory factor IL-6 by vascular endothelial cells in diabetes [12].

OMT is an alkaloid extracted from *Sophora flavescens* Ait, which has shown anti-inflammatory, antitumor, antiviral, and anti-arrhythmic effects [30, 31]. Thus its anti-

Fig. 10. Effect of OMT or A_{2B} siRNA on phosphorylation of ERK1/2 in HUVECs cultured in high glucose medium. ERK1/2 and P-ERK1/2 at the protein level were detected by Western blotting and the stain values (integrated optical density) of P-ERK protein expression (normalized to each ERK 1/2) were quantitated using image analysis. Values are the mean $\pm$ SEM of 6 observations. ** $p < 0.01$ vs 22.2 mM group or 22.2mM + scramble siRNA.

inflammatory and vasodilating effects might relieve vascular endothelial injury induced by high glucose and high lipids. In this study, homology modeling and molecular docking analysis disclosed that OMT can interact with the A_{2B} receptor. Our results have also shown that high glucose can significantly increase the expression of the A_{2B} receptor in HUVECs, suggesting that the A_{2B} receptor may be associated with diabetic vascular endothelial dysfunction. This is consistent with the observations by Figler et al. [12].

Our current study also confirmed that the expression of the chemokine CCL5 in HUVECs was significantly increased in response to glucotoxicity. CCL5 has long been considered as a potential target to reduce macrophage infiltration into the artery wall because CCL5 can result in a localized, potent chemotactic signal [32]. Thus high glucose may increase the expression and release of chemokines and inflammatory cytokines by activating A_{2B} receptors, leading to vascular inflammation and damage of vascular endothelial function via promoting the adhesion and migration of immune cells. OMT intervention could effectively suppress the increased expression of A_{2B} receptor and CCL5 in HUVECs in the high-glucose culture.

Vascular endothelial dysfunction is the basis of diabetic vascular complications, and chronic exposure to high glucose reduces nitric oxide (NO) production from the endothelium [33]. The ultimate goal of treatment for patients with diabetes is to prevent and delay a variety of chronic complications. Improvement of endothelial cell function could be one of the effective approaches in this regard. This study showed that high-glucose culture for 3 days reduced HUVEC viability and NO production, but the damage could be significantly relieved after intervention with OMT or A_{2B} siRNA. This study has revealed that the expressions of pro-inflammatory genes including TNF- α , IL-6 and IL-1 β were increased by LPS-induced inflammatory response in HUVECs [34]. In this study, the results also revealed that IL-1 β and TNF- α levels increased significantly due to high glucose, and that OMT could reverse

Fig. 11. Effect of OMT or A_{2B} siRNA on phosphorylation of p38 MAPK in HUVECs cultured in glucose medium. P-p38 and p38 MAPK at the protein level were detected by Western blotting. Using image analysis, the stain values (integrated optical density) of P-p38 MAPK (normalized to each p38 MAPK) were obtained. Values are the mean ± SEM of 6 observations. **p<0.01 vs 22.2 mM group or 22.2mM + scramble siRNA.

the change. Meanwhile A_{2B} siRNA treatments had similar effects to OMT. All of these results indicate that OMT can protect human vascular endothelial cells from high glucose-induced toxicity possibly through interfering with the A_{2B} receptor and decreasing the expression of CCL5, IL-1β and TNF-α.

To further explore the molecular mechanism underlying the protective effect of OMT on HUVECs from high glucose toxicity, we examined the phosphorylation of ERK1/2 and p38, which control two classic MAPK pathways implicated in the responses to cell stress, inflammation and glucotoxicity. Many studies have proven that MAPKs can be activated in HUVECs by high glucose and may play key roles in the regulation of high glucose-induced inflammatory cytokine expression [3, 35]. The results showed that high glucose culture significantly increased both P-ERK1/2 and P-p38 without altering total ERK1/2 and p38 in HUVECs. Moreover, co-treatment with OMT could significantly diminish the upregulated P-ERK1/2 and P-p38 due to high glucose exposure of HUVECs. Meanwhile the A_{2B} siRNA treatments also had similar effects to OMT. These observations point to the important implication of activation of classical ERK1/2 and p38 MAPK pathways in high-glucose toxicity and a potential mechanism of how OMT may alleviate glucotoxicity.

Conclusion

OMT has protective effects on HUVECs against high-glucose toxicity. Glucotoxicity can increase the expression of the A_{2B} receptor in HUVECs. OMT can decrease the expression of the A_{2B} receptor in the diabetic cell model and inhibit the release of pro-inflammatory mediators such as inflammatory cytokines (CCL5) and subsequent activation of p38 and ERK1/2 MAPK signaling pathways to alleviate the toxic effects of high glucose.

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Disclosure Statement

All authors declare that they have nothing to disclose.

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