

## Tanshinone IIA inhibits AGEs-induced proliferation and migration of cultured vascular smooth muscle cells by suppressing ERK1/2 MAPK signaling

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### ARTICLE INFO

#### Article type:

Original article

#### Article history:

Received: Nov 13, 2016

Accepted: Sep 28, 2017

#### Keywords:

Advanced

ERK1/2

Glycation end products

JNK

P38

Tanshinone IIA

Vascular Smooth Muscle

### ABSTRACT

**Objective(s):** Vascular smooth muscle cells (VSMCs) play a key role in the pathogenesis of diabetic vascular disease. Our current study sought to explore the effects of tanshinone IIA on the proliferation and migration of VSMCs induced by advanced glycation end products (AGEs).

**Materials and Methods:** In this study, we examined the effects of tanshinone IIA by cell proliferation assay and cell migration assay. And we explored the underlying mechanism by Western blotting.

**Results:** AGEs significantly induced the proliferation and migration of VSMCs, but treatment with tanshinone IIA attenuated these effects. AGEs could increase the activity of the ERK1/2 and p38 pathways but not the JNK pathway. Treatment with tanshinone IIA inhibited the AGEs-induced activation of the ERK1/2 pathway but not the p38 pathway.

**Conclusion:** Tanshinone IIA inhibits AGEs-induced proliferation and migration of VSMCs by suppressing the ERK1/2 MAPK signaling pathway.

#### ► Please cite this article as:

Lu M, Luo Y, Hu P, Dou L, Huang SH. Tanshinone IIA inhibits AGEs-induced proliferation and migration of cultured vascular smooth muscle cells by suppressing ERK1/2 MAPK signaling. Iran J Basic Med Sci 2018; 21:83-88. doi: 10.22038/IJBMS.2017.20100.5276

### Introduction

The increasing incidence of the diabetes mellitus, an insulin-mediated syndrome associated with abnormal metabolism, has made it a major threat to human health (1). Patients with diabetes mellitus have an increased risk of cardiovascular complications such as atherosclerosis (AS), resulting in high mortality and morbidity (2, 3). AS is the main form of a group of atherosclerotic vascular disease. AS begins from the arterial intima and spreads to the muscular layer of the large and medium arteries, eventually leading to the obstruction of the vascular lumens. AS involves a series of pathophysiological processes that include endothelial damage, the infiltration of oxidized low-density lipoprotein and an abnormal proliferation of vascular smooth muscle cells (VSMCs)(4, 5). A high blood glucose level resulting from a deficiency in or resistance to insulin secretion has been assumed to induce AS (6). A high blood glucose level not only contributes to the proliferation and DNA synthesis of VSMCs but can also increase their migration potential (7, 8).

Following the persistent exposure to high blood glucose, advanced glycation end products (AGEs) are produced by the Maillard reaction, a non-enzymatic and irreversible process. With the accumulation of AGEs and their interactions with their receptors, endothelial cells are inclined to undergo apoptosis and VSMCs are stimulated to proliferate, facilitating the development of AS in diabetic patients (9-11). A great deal of research has been performed to clarify the specific mechanisms underlying this process. It was previously performed to show that the proliferation and migration of VSMCs is associated with the generation of reactive oxygen species (ROS)(12), a reduction in the bioavailability of NO (13), and reduction in many cytokines and growth factors such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and cell adhesion molecule-1 (VCAM-1)(14,15).

Tanshinone IIA is the most active diterpenoid quinone pigment in danshen, which is a crucial source of a variety of active natural compounds. Tanshinone IIA has been shown to have beneficial effects on diabetes by other studies. For example, it could decrease body

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weight, improve glucose tolerance and reduce the low density lipoprotein to high-density lipoprotein ratio (16). Moreover, treatment with tanshinone IIA could attenuate VSMCs proliferation and intimal hyperplasia (17). It has been suggested that this beneficial effect on the VSMCs is the consequence of the activation of an AMP-activated protein kinase pathway (18, 19). However, despite the number of studies that have been performed on the topic, there is still little known about the influence of tanshinone IIA on the AGEs-induced activation of VSMCs. Hence, in our present study, we explored the effects of tanshinone IIA on the AGEs-induced proliferation and migration of VSMCs and its potential mechanism of action.

## Materials and Methods

### Cell culture

Primary vascular smooth muscle cells were isolated from the thoracic aortas of Sprague-Dawley rats (5-8 weeks old) as described previously (21). The VSMCs were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) at 37 °C with 5% CO<sub>2</sub>. Only VSMCs from passages 4-6 were used for our experiments. Immunofluorescent staining with monoclonal anti-smooth muscle actin antibody was used for the identification and characterization of VSMCs.

### Preparation of AGEs

AGEs were prepared as described previously (20). BSA was incubated with 0.5 M glucose in phosphate-buffered saline (PBS) in the dark for 16 weeks at 37 °C. The unincorporated sugars were removed by dialyzing against PBS (pH 7.4). Control nonglycated BSA was incubated in the absence of glucose under the same conditions. The endotoxin levels were checked using an endotoxin testing kit (Chromogenic TAL Endpoint Assay Kit, Xiamen, Fujian, China). The AGE-BSA solutions were confirmed to be endotoxin-free (<2.5U/ml of endotoxin).

### Drugs and chemicals

Tanshinone IIA was purchased from Shanghai RongHe Bioengineering Company (Shanghai, China). The ERK1/2 inhibitor (PD 98059), p38 inhibitor (SB203580) and FITC-conjugated monoclonal anti- $\alpha$ -smooth muscle actin antibody were obtained from Sigma Chemical Company (St. Louis, MO, USA). Trypsin, FBS and DMEM were purchased from Gibco Chemical Company (Grand Island, NY, USA). Primary antibodies, including rabbit anti-p-ERK1/2, rabbit anti-p-p38, rabbit anti-p38, rabbit anti-p-JNK, rabbit anti-JNK and goat anti-rabbit secondary antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). The rabbit anti-ERK1/2 antibody was obtained from Santa Cruz Biotechnology (CA, USA). Cell migration was evaluated using a 24-well transwell plate that contained polycarbonate 8- $\mu$ m pore membrane filters (Milipore, Bedford, MA, USA).

### Cell proliferation assay

After serum-free culture for 24 hr, the VSMCs were seeded at 6,000 cells per well in 96-well culture plates. After being treated with AGEs or AGEs supplemented with tanshinone IIA for 48 hr, 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to the cells for 4 hr at 37 °C according to the manufacturer's instructions. The absorbance was examined at a wavelength of 570 nm.

### Scratch assay

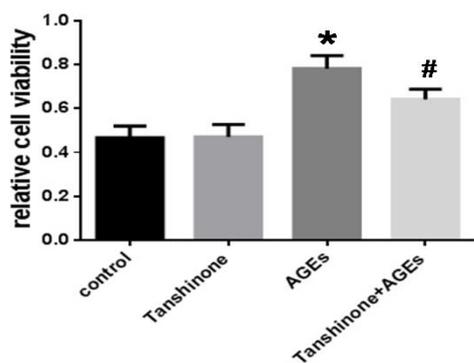
VSMCs were grown in 6-well plates then the confluent monolayers were scratched with 200- $\mu$ l pipette tips. The cells were rinsed twice then the size of the denuded surface was measured. The VSMCs were subsequently incubated with serum-DMEM containing different drugs and AGEs for 24 hr. Next, the area of the cells which had migrated across the denuded area was measured (21).

### Transwell migration assay

The migration of VSMCs was examined using a 24-well transwell plate which contained gelatin-coated polycarbonate membrane filters. The VSMCs were harvested using 0.25% trypsin then seeded in the upper wells (at 10,000 cells in 200  $\mu$ l serum-free DMEM containing 1% FBS), while the lower wells were filled with DMEM containing 10% FBS. The AGEs and drugs were added to the upper wells, and then, the cells were allowed to migrate for 12 hr at 37 °C. Non-migratory cells were removed from the upper surface of the membrane by scraping with cotton swabs. The membrane was stained with DAPI (1.4  $\mu$ mol/l) and was evaluated using wide-field fluorescent microscopy (Zeiss) (21).

### Western blot analysis

After various treatments, the cells were washed twice with cold PBS and lysed on ice for 20 min in lysis buffer [50 mmol/l Tris-HCl (pH 7.5), 150 mmol/l NaCl, 1% Nonidet P-40, 0.5% sodiumdeoxycholate, 0.1% SDS, and 1 mmol/l EDTA] containing a protease inhibitor cocktail (1 mmol/l PMSF, 1 mmol/l Na<sub>3</sub>VO<sub>4</sub> and 10 mmol/l NaF). Whole cell lysates were prepared by scraping cells from 6-well plates and the proteins were quantified with a Bio-Rad DC protein assay kit. Samples containing equal amounts of protein were electrophoresed on 10% SDS-polyacrylamide gels and transferred to PVDF membranes. Membranes were blocked with 5% non-fat milk in TBST buffer (50 mM Tris, pH 7.5, 250 mM NaCl, 0.1% Tween 20) for 1 hr at room temperature and then were incubated with primary antibodies overnight at 4 °C. After being washed three times with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hr at room temperature. Finally, the membranes were incubated with enhanced chemiluminescence (ECL) reagents and the immunoreactive bands were visualized on a LAS-4000 imaging system (Fujifilm, Tokyo, Japan).



**Figure 1.** VSMCs showed faster proliferation following exposure to AGEs (100 µg/ml) for 48 hr. The AGEs-induced proliferation of VSMCs was inhibited by pretreatment with tanshinone II A (10 µmol/l) for 1 hr. \* $P < 0.05$  vs control; # $P < 0.05$  vs AGEs. values = mean ± SEM (n= 3) VSMCs: Vascular smooth muscle cells; AGEs: advanced glycation end products

### Statistical analysis

All of the experiment results are expressed as the mean values ± SD. The statistical analysis was performed with a one-way analysis of variance for multiple comparisons using the SPSS version 16.0 software program. A value of  $P < 0.05$  was considered statistically significant.

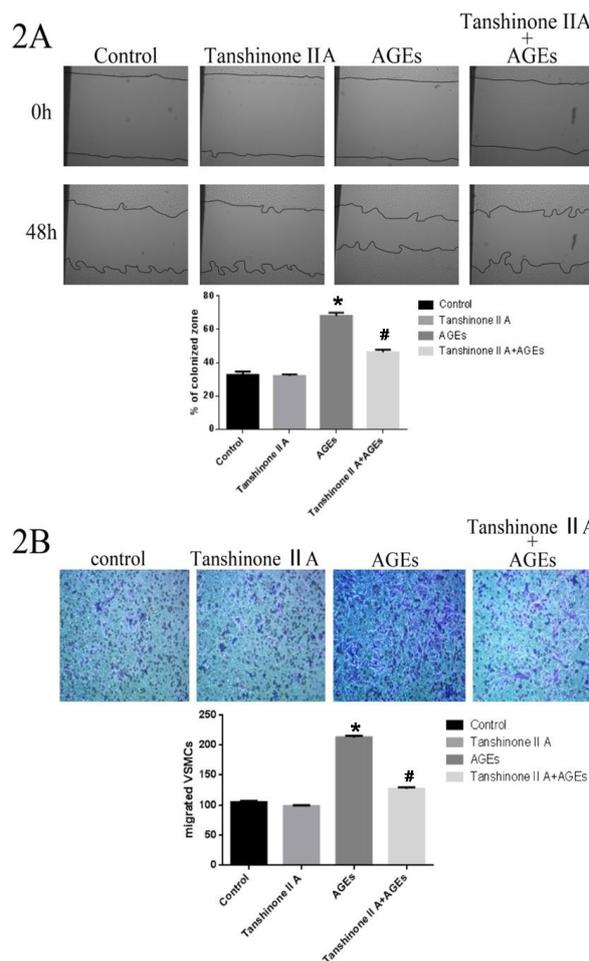
## Results

### Tanshinone IIA inhibited the AGEs-induced proliferation of VSMCs

To explore the effects of tanshinone IIA on the AGEs-exposed VSMCs, we first evaluated the influence of AGEs on the proliferation of VSMCs. The results suggested that VSMCs showed higher proliferation when exposed to AGEs (100 µg/ml) for 48 hr compared to those without AGEs exposure. The VSMCs were then exposed to tanshinone IIA (10 µmol/L) for 1 hr and subsequently treated with or without AGEs for 48 hr. We found that the AGEs-induced proliferation of VSMCs was inhibited by pretreatment with tanshinone IIA (Figure 1). These results demonstrate that tanshinone IIA could inhibit the AGEs-induced proliferation of VSMCs.

### Tanshinone IIA inhibited the AGEs-induced migration of VSMCs

A scratch assay and a transwell migration assay were applied to assess the migratory potential of VSMCs when they were exposed to AGEs. Incubation with AGEs (100 µg/ml) enhanced the closure of a linear scratch compared to the control group (Figure 2A). The same effect was observed in the transwell assay. As shown in Figure 2B, the number of cells on the lower side of the membrane was increased following AGEs treatment compared to the control cells over a 12 hr period. Hence, AGEs exposure could induce VSMCs migration. In contrast, tanshinone IIA abrogated the AGEs-induced migration at a concentration of 10 µmol/l. Thus, the AGEs-induced migration of VSMCs can be attenuated by tanshinone II A.



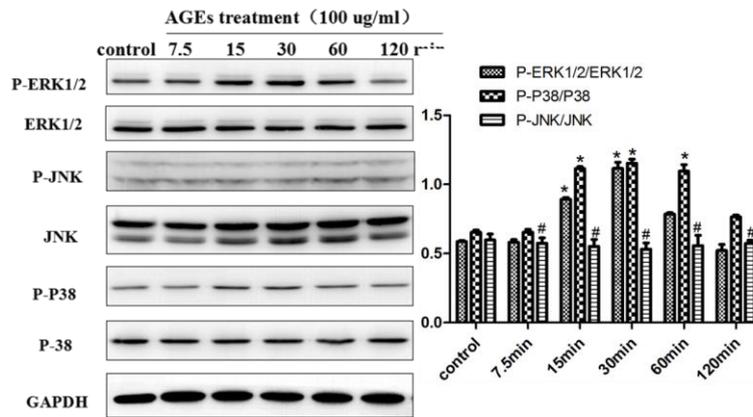
**Figure 2.** A: The wound healing assay. Incubation of VSMCs with AGEs (100 µg/ml) enhanced the closure of a linear scratch line. Tanshinone IIA pretreatment (10 µmol/l) abrogated the AGEs-induced migration. \* $P < 0.05$  vs control; # $P < 0.05$  vs AGEs. Values = mean ± SEM (n=3). B: The transwell migration assay. The number of cells on the lower side of the membrane increased following exposure to AGEs. In contrast, tanshinone IIA abrogated the AGEs-induced migration at a concentration of 10 µmol/l. \* $P < 0.05$  vs control; # $P < 0.05$  vs AGEs. values = mean ± SEM (n=3) VSMCs: Vascular smooth muscle cells; AGEs: advanced glycation end products

### Exposure of VSMCs to AGEs activated the ERK1/2 and p38 MAPK pathways

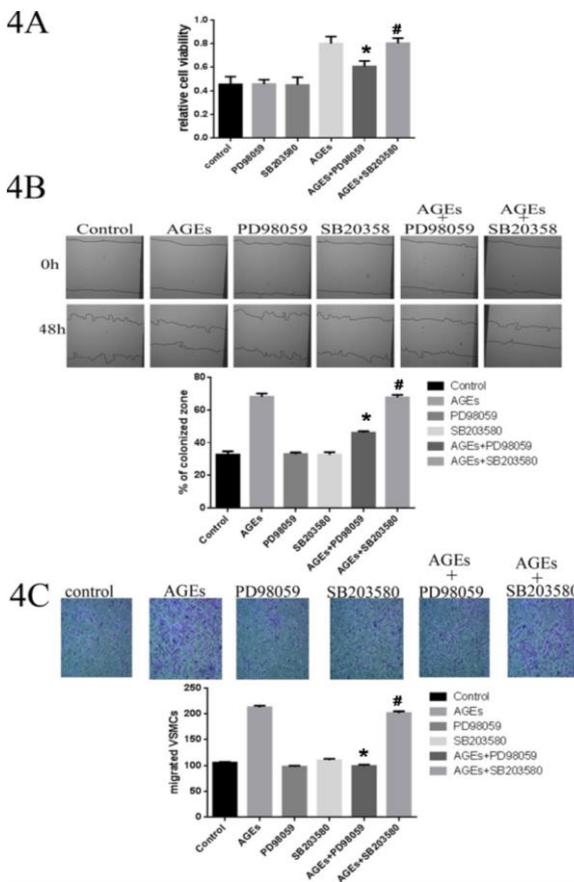
We next evaluated the MAPK signaling pathway to explore the possible mechanism by which the proliferation and migration of VSMCs was induced by AGEs. The VSMCs were incubated with AGEs (100 µg/ml) for different lengths of time. The exposure of VSMCs to AGEs activated ERK1/2 and p38 MAPK signaling but not JNK MAPK signaling. The results are shown in Figure 3.

### The ERK1/2 inhibitor, PD98059, inhibited the AGEs-induced proliferation and migration of VSMCs

To further investigate whether the ERK1/2 and p38 MAPK signaling pathways are associated with the AGEs-induced proliferation and migration of VSMCs, we assessed the effects of an ERK1/2 inhibitor and a p38



**Figure 3.** VSMCs were incubated with 100 µg/ml AGEs for the indicated lengths of time. The exposure of VSMCs to AGEs activated ERK1/2 and p38 MAPK signaling but not JNK MAPK signaling. \**P*<0.05 vs control; values = mean±SEM (n = 3)

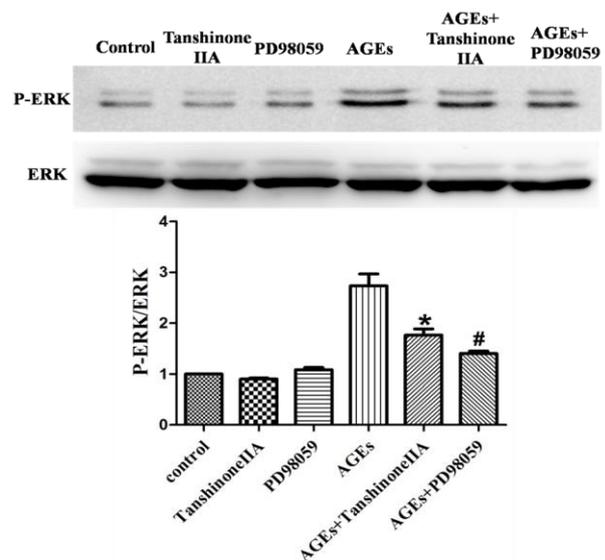


**Figure 4.** A: The ERK1/2 inhibitor, PD98059, inhibited the AGEs-induced proliferation of VSMCs. The VSMCs were exposed to PD98059 (25 µmol/l) or a p38 inhibitor, SB203580, for 1 hr before AGEs treatment. \**P*<0.05 vs AGEs; #*P*<0.05 vs AGEs+PD98059. values=mean ±SEM (n=3). B: The ERK1/2 inhibitor, PD98059, inhibited the AGEs-induced migration of VSMCs in the wound healing assay. The VSMCs were exposed to the ERK1/2 inhibitor PD98059 (25 µmol/l) or the p38 inhibitor, SB203580, for 1 hr before AGEs treatment. \**P*<0.05 vs AGEs; #*P*<0.05 vs AGEs+PD98059. values = mean ± SEM (n=3). C: The ERK1/2 inhibitor, PD98059, inhibited the AGEs-induced migration of VSMCs in the transwell migration assay. The VSMCs were exposed to the ERK1/2 inhibitor, PD98059 (25 µmol/l), or the p38 inhibitor, SB203580, for 1 hr before AGEs treatment. \**P*<0.05 vs AGEs; #*P*<0.05 vs AGEs+PD98059. values = mean ± SEM (n=3)

inhibitor on VSMCs cultured with AGEs. The VSMCs were exposed to an ERK1/2 inhibitor, PD98059 (25 µmol/l) or a p38 inhibitor, SB203580 (10 µM), for 1 hr before AGEs treatment (22). Only the ERK1/2 inhibitor could attenuate the AGEs-induced proliferation and migration of VSMCs. These results are shown in Figures 4 A-C.

**Tanshinone IIA inhibited AGEs-induced ERK1/2 MAPK signaling**

To determine the interaction between tanshinone IIA and ERK1/2 MAPK signaling, we examined the effects of tanshinone IIA and an ERK1/2 inhibitor. The VSMCs were exposed to tanshinone IIA (10 µmol/l) and an ERK1/2 inhibitor, PD98059 (25 µmol/l, for 1 hr before being treated with AGEs for 30 min. As shown in Figure 5, tanshinone IIA inhibited the AGE-induced activity of ERK1/2 MAPK.



**Figure 5.** The VSMCs were exposed to tanshinone IIA (10 µmol/l) and an ERK1/2 inhibitor, PD98059 (25 µmol/l) for 1 hr before being treated with AGEs for 30 min. \**P*<0.05 vs AGEs; #*P*<0.05 vs AGEs. values = mean ± SEM (n=3)

## Discussion

As one of the most common complications of diabetes mellitus, atherosclerosis has been considered the primary factor responsible for the mortality and morbidity of diabetes patients (23). The accumulation of VSMCs is critical in the pathogenesis and progression of microvascular diseases. The excessive proliferation of VSMCs which arises from high blood glucose levels has been considered the initial factor that accelerates the occurrence and development of AS (24).

Recent studies have indicated that AGEs play an important role in AS via the induction of VSMCs proliferation (25, 26). The results of our present study are consistent with the previous findings. We found that the VSMCs exhibited enhanced proliferation and migration when exposed to AGEs. However, the underlying mechanisms by which AGEs induce these changes in VSMCs are not fully understood. YW Yoon *et al.* verified that AGEs promote VSMCs proliferation via the ERK and p38 MAPK pathways (27). In addition to the MAPK pathway, p21, NF- $\kappa$ B and JAK/STAT are also apparently involved in the process (28, 29). Other studies have demonstrated that the proliferation of VSMCs is related to a variety of cytokines and growth factors, including platelet-derived growth factor and basic fibroblast growth factor (30). The results of our present study correlated well with those of previous studies showing that the ERK1/2 and p38 MAPK signaling pathway were activated by AGEs.

To clarify whether the activation of the ERK1/2 and p38 MAPK signaling pathways is the cause of the AGEs-induced proliferation and migration of VSMCs, we used the ERK1/2 inhibitor, PD98059, and the p38 inhibitor, SB203580, to treat VSMCs before they were exposed to AGEs. The results demonstrated that the ERK1/2 inhibitor, but not the p38 inhibitor, could attenuate the AGEs-induced proliferation and migration of VSMCs. Therefore, the ERK1/2 MAPK signaling plays an important role in regulating the proliferation and migration of VSMCs.

Tanshinone IIA, which is extracted from Danshen, is widely applied in clinical practice because of its various cardiovascular activities (31). Tanshinone IIA has protective effects on the early stage of diabetic nephropathy (32). Earlier studies suggested that tanshinone IIA could improve glucose tolerance, insulin sensitivity and glucose metabolic disorders by activating AMPK signaling (33). However, there have been no previous studies that have identified the effects of tanshinone IIA on the AGEs-induced proliferation and migration of VSMCs. The data from our present research shows that the AGEs-induced proliferation and migration of VSMCs were greatly attenuated by tanshinone IIA treatment. We further explored the specific mechanism, and found that the ERK1/2 MAPK signaling pathway was markedly inhibited by tanshinone IIA pretreatment, even when the cells were subsequently exposed to AGEs. These findings

demonstrated that tanshinone IIA had protective effects against AGEs-induced changes in VSMCs by suppressing ERK1/2 MAPK signaling.

Our study is particularly important because it demonstrates how tanshinone IIA affects the AGEs-induced dysfunction in VSMCs. Further studies will be required to discover how tanshinone IIA activates the ERK1/2 MAPK signaling pathway. The current results will also need to be confirmed in animal studies.

## Conclusion

We herein provided experimental evidence that tanshinone IIA can inhibit the AGEs-induced proliferation and migration of VSMCs. We also demonstrated that the ERK1/2 MAPK signaling pathway is involved in the beneficial effects of tanshinone IIA on the activation of VSMCs induced by AGEs. In summary, tanshinone IIA inhibits the AGEs-induced proliferation and migration of VSMCs by suppressing the ERK1/2 MAPK signaling pathway. Therefore, tanshinone IIA may represent a potential candidate for preventing diabetic atherosclerosis.

## Acknowledgment

This work was supported by grants from Science Foundation of Health Bureau of Zhejiang Province (2015KYB273, P.F. Hu) and the Natural Science Foundation of Zhejiang Province (Y13H020030, S.W. Huang). All research was performed in the Biomedical Research Centre, Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, Hangzhou, China.

## Conflict of Interest

The authors declare no conflict of interest.

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