Salvia miltiorrhiza Depresses Plasminogen Activator Inhibitor-1 Production Through Inhibition of Angiotensin II

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Abstract: The purpose of this study was to investigate the effect of Salvia miltiorrhiza on the production of plasminogen activator inhibitor-1 (PAI-1) induced by angiotensin II (Ang II) in renal mesangial cells. Rat mesangial cells were exposed to 100 nM Ang II. Meanwhile, different concentrations of Salvia miltiorrhiza injection were added to Mesangial Cells. PAI-1 mRNA was measured by semi-quantification reverse transcriptase polymerase chain reaction (RT-PCR) and PAI-1 protein by Western blotting. ELISA was used to detect the expression of transforming growth factor β1 (TGF-β1) in serum free MEM medium. The level of cellular reactive oxygen species (ROS) was measured by confocal laser scanning microscopy. Salvia miltiorrhiza notably attenuated expression of PAI-1 induced by Ang II in a concentration-dependent manner. Meanwhile, it suppressed the production of TGF-β1 and cellular ROS in mesangial cells. These effects were due to Salvia miltiorrhiza’s ability of inhibiting the activities of angiotensin II. Therefore, Salvia miltiorrhiza can be used to retard progression of glomerular sclerosis.

Keywords: Salvia miltiorrhiza; Transforming Growth Factor β1; Plasminogen Activator Inhibitor Type 1; Reactive Oxygen Species.

Introduction

Renal glomerular and interstitial fibrosis is viewed as a final event leading to renal failure, regardless of the initiating injury. Accumulation of extracellular matrix (ECM)
leads to glomerulosclerosis, which is a central feature of progressive glomerular disease. Although enhanced ECM synthesis has been demonstrated to contribute to ECM accumulation, decreased degradation of the ECM could also play an important role in matrix accumulation and glomerulosclerosis. Two major ECM protease systems, plasminogen activator (PA)/plasmin/PA inhibitors (PAI) system and matrix metalloproteinases (MMP)/tissue inhibitors of matrix metalloproteinases (TIMP) system, are interrelated and involved in matrix degradation (Stetler-Stevenson, 1996). Plasminogen activator inhibitor-1 (PAI-1), a principal inhibitor of plasminogen activators (tissue-type plasminogen activator and urokinase-type plasminogen activator), can promote glomerulosclerosis by preventing the activation of matrix metalloproteinases (MMPs) and the degradation of ECM by plasminogen activators and plasmin (Eddy, 2002).

Angiotensin II (Ang II), the major effector molecule of the renin-angiotensin system (RAS), has been perceived as a potent fibrotic factor responsible for the accumulation of extracellular matrix (Ma et al., 2004). The standard renoprotection is based on the inhibition of RAS by angiotensin convertase inhibitors (ACEi) or angiotensin II receptor 1 blockers (AT1B) (Litwin et al., 2006). All of the components of RAS are present within the glomerulus where they may function locally to produce Ang II and Ang II type 1 receptor is strongly expressed in mesangial cells. Therefore, locally produced Ang II can act on mesangial cells, the key producers of extracellular matrix in progressive glomerulosclerosis (Jaimes et al., 2005). Ang II can act through increasing the synthesis of PAI-1 to promote glomerulosclerosis (Kagami et al., 1997). Regression of glomerulosclerosis with high-dose angiotensin inhibition is linked to decreased plasminogen activator inhibitor-1 (Ma et al., 2005).

Salvia miltiorrhiza, root of Salvia miltiorrhiza BGE, is officially listed in the Chinese Pharmacopeia and is commonly used in mainland China for the treatment of atherosclerosis-related disorders such as cardiovascular and cerebrovascular diseases. It is also proven that Salvia miltiorrhiza can significantly ameliorate hepatic fibrosis. Salvia miltiorrhiza exerts antifibrotic effects by several mechanisms, including down-regulating of several fibrogenic chemokines (Wasser et al., 1998; Lee et al., 2003; Hsu et al., 2005), inducting apoptosis in hepatic stellate cells (Lee et al., 2006) and reducing oxidant stress (Lee et al., 2003).

Recently, Salvia miltiorrhiza is used to treat renal disease (Wojcikowski et al., 2006). Kang et al. (2002) have demonstrated that Salvia miltiorrhiza has ACE-inhibitory activities. Furthermore, Xu et al. (2001) suggested that Salvia miltiorrhiza can inhibit mesangial cell proliferation. Salvanianolic acid B (Sal B), a pure water-soluble compound extracted from Salvia miltiorrhiza, has been reported to attenuate PAI-1 production in TNF-a treated human umbilical vein endothelial cells (Zhou et al., 2005). However, it is not yet clear whether the treatment of Salvia miltiorrhiza can decrease PAI-1 production through inhibiting the activities of angiotensin II and then ameliorate glomerulosclerosis.

To clarify this hypothesis, we examined the changes of PAI-1, TGF-β1 expression and cellular reactive oxygen species level induced by Ang II in mesangial cells after being treated with Salvia miltiorrhiza injection.
SALVIA MILTIORRHIZA DEPRESSES PAI-1 IN MESANGIAL CELLS

Materials and Methods

Salvia miltiorrhiza Injection

Salvia miltiorrhiza injection is a solution containing danshensu and other water-soluble active components of Salvia miltiorrhiza. It is purified by a series of refining processes, such as water extraction, ethanol precipitation and ultrafiltration concentration. The injection has been used clinically for intramuscular or intravenous injection in China for the last 20 years. In this experiment, Salvia miltiorrhiza injection (No. 0508242) was purchased from Chiatai Qingchunbao Pharmaceutical Corporation (Hangzhou, China).

Cell Culture

The rat mesangial cell line (HBZY-1) purchased from China Center for type culture collection (Wuhan, China) was plated in MEM containing 10% fetal bovine serum (FBS, Gibco), 15 mM N-2-hydroxy-ethylpiperazine-N′-2-ethane-sulfonic acid (HEPES), 20 mM NaHCO3, 100 U/ml penicillin, 100 µg/ml streptomycin, and cultured in a humidified atmosphere of 95% air and 5% CO2 at 37°C. Cells were cultured in 100-mm culture dishes for Western blot measurement, on cover glasses coated with polylysine for intracellular ROS measurement, and 6-well culture plates for enzyme-linked immunosorbent assay (ELISA) and RT-PCR. Near-confluent mesangial cells were incubated with serum-free media for 24 hours to arrest and synchronize the cell growth. After this period, the media was changed to fresh serum-free MEM containing different concentrations of Salvia miltiorrhiza injection or angiotensin II (Sigma, St Louis, MO, USA), or in combination for 24 hours. Cells were processed to measure ROS level, TGF-β1 expression, and PAI-1 mRNA and protein expression at the end of the incubation period as described below.

Reverse Transcriptase Polymerase Chain Reaction

RT-PCR was performed to determine the relative quantity of PAI-1 mRNA in mesangial cells, whereas GAPDH mRNA, a housekeeping gene, was used as an internal control. Briefly, total RNA was extracted from mesangial cells with Trizol (Invitrogen Corporation, California, USA). First-strand cDNA synthesis was reversely transcribed from 2 µg of mRNA in transcription buffer and 200U MMLV reverse transcriptase (Promega, WI, USA) at 42°C for 1 hour, followed by immediate cooling on ice. PCR amplification was performed by using Taq polymerase (Takara, Shiga, Japan) in a total volume of 50 µl. The upstream and downstream primers for rat PAI-1 mRNA were 5'-GCC TCC AAA GAC CGA AAT GTG-3' and 5'-GTC GTT GAT GAT GAA TCT GGC TC-3', yielding a 317 bp product, whereas those for GAPDH were 5'-TAG CCC AGG ATG CCC TTT AGT-3' and 5'-CCC CCA ATG TAT CCG TTG TG-3', yielding a 119 bp product. PCR conditions were as follows: 35 cycles, denaturing at 94°C for 30 sec, annealing at 52°C for 60 sec and extending at 72°C for 60 sec with initial heating at 94°C for 3 min and final extending at 72°C for 10 min. The PCR products were separated by 1.5% agarose electrophoresis and the band density images were captured, and the signals were quantified in arbitrary units as OD × band area using
Western Blot Analysis

Western blot analysis was performed to determine the secreted PAI-1 in the mesangial cell conditioned media. The media was cleared by centrifugation. Aliquots of cleared media normalized for cell number were concentrated in a free dry system (Labconco, USA). Protein concentration was determined by using a protein assay kit (Pierce, Rockford, IL, USA). Total proteins (50 µg) were electrophoresed through standard 12% SDS-PAGE in tris-glycine electrophoresis buffer [25 mm Tris, 192 mM glycine (pH 8.3), and 0.1% SDS] and blotted onto nitrocellulose membrane in transferring buffer [380 mM glycine, 50 mM Tris (pH 8.3) and 20% methanol] at 80 mA for 2 hours in a water-cooled transfer apparatus. The membrane was pre-incubated in blocking buffer (TBS containing 5% non-fat dried milk) for 2 hours at room temperature and then incubated with polyclonal anti-PAI-1 antibody (1:200 diluted in the blocking buffer, Santa Cruz) overnight at 4°C. The membrane was washed 3 times with 0.1% Tween-PBS, then it was incubated with rabbit anti-goat IgG conjugated with horseradish peroxidase (Santa Cruz, CA, USA) diluted to 1:2000 in the blocking buffer for 2 hours at room temperature. Antibody-antigen complexes were detected by chemiluminescence by using enhanced chemiluminescent substrate kit (Santa Cruz, CA, USA). Images were captured on a X-ray film, and the signals were quantified in arbitrary units (OD × band area) using Vilber Lourmat image analysis system (Vilber Lourmat, Marne-la-Vallee Cedex 1, France).

Intracellular ROS

The intracellular formation of ROS was detected by confocal microscopy using the fluorescent probe 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H$_2$DCFDA; Molecular Probes Inc., Eugene, OR), as described by Lee et al. (2005). In brief, cover glasses of confluent cells in different groups were washed with Dulbecco’s phosphate-buffered saline (PBS) and incubated in the dark for 45 min in PBS containing 10 mM CM-H$_2$DCFDA at 37°C. Subsequently, the cells were washed 3 times with PBS to remove any excess dye. CM-H$_2$DCFDA, which is oxidized to the highly fluorescent 2′,7′-dichlorofluorescein (DCF) by H$_2$O$_2$ or OH, was used as a marker for ROS generation. Cells were viewed by using 40 × 1.4 objective lens equipped with a laser scanning confocal attachment (Leica, Wetzlar, Germany). CM-H$_2$DCFDA was excited with the 488-nm line of an argon/krypton mixed gas laser. Emission was collected with a 510-nm long pass filter. Images were obtained using the identical microscope settings, such as contrast and brightness, for all samples. The fluorescent images were quantified by Leica Confocal Software program.

ELISA Assay

TGF-β$_1$ in conditioned media was quantitatively measured by ELISA method according to the manufacturer’s instructions for the TGF-β$_1$-ELISA Detection Kit (Boster, Wuhan, China).
Statistical Analysis

All results are expressed as means ± standard error (SE). Analysis of variance was used to assess the differences between multiple groups. If the F statistics was significant, the mean values obtained from each group were then compared by Fisher’s least significant difference method. \( p < 0.05 \) was used as the criterion for a statistically significant difference.

Results

Effects of Salvia miltiorrhiza on Ang II-Induced PAI-1 mRNA Expression and Protein Secretion in Mesangial Cells

To examine the effects of Salvia miltiorrhiza on Ang II-induced PAI-1 mRNA expression and protein secretion, mesangial cells were cultured with or without Salvia miltiorrhiza injection (37.5 mg/ml, 75 mg/ml, or 150 mg/ml) in the presence of Ang II (100 nM) for 24 hours. The effect of Salvia miltiorrhiza on Ang II-induced PAI-1 mRNA expression was analyzed by RT-PCR (Fig. 1). Ang II at 100 nM up-regulated PAI-1 mRNA expression by 1.9-fold compared to the control at 24 hours. Salvia miltiorrhiza inhibited Ang II-induced PAI-1 mRNA expression dose dependently in mesangial cells. Salvia miltiorrhiza alone did not affect basal PAI-1 mRNA expression.

![Figure 1](image_url)

Figure 1. Induction of PAI-1 mRNA with Ang II and/or Salvia miltiorrhiza in mesangial cells. (A) Typical RT-PCR results. Lane 1: marker; lane 2: untreated control; lane 3: treatment with 100 nM Ang II; lane 4: treatment with Ang II (100 nM) + Salvia miltiorrhiza injection (37.5 mg/ml); lane 5: treatment with Ang II (100 nM) + Salvia miltiorrhiza injection (75 mg/ml); lane 6: treatment with Ang II (100 nM) + Salvia miltiorrhiza injection (150 mg/ml); lane 7: treatment with Salvia miltiorrhiza injection (150 mg/ml). (B) Relative mRNA level of PAI-1, which are normalized with GAPDH levels. \( * p < 0.05 \) vs. control; \( ** p < 0.01 \) vs. control; \( \# p < 0.05 \) vs. group treated by Ang II; \( ## p < 0.01 \) vs. group treated by Ang II.
After 24-hour incubation, Ang II increased PAI-1 protein secretion by 3-fold compared to the control group. However, Salvia miltiorrhiza dose dependently inhibited Ang II-induced PAI-1 protein secretion. Salvia miltiorrhiza alone did not inhibit basal level of PAI-1 protein secretion (Fig. 2).

**Effect of Salvia miltiorrhiza on Cellular ROS in Mesangial Cells**

Intracellular DCF-sensitive ROS was visualized by a confocal microscopy, and the results were analyzed by Leica confocal software program. After mesangial cells were incubated with 100 nM Ang II for 24 hours, ROS level in cells increased significantly by 4.6-fold compared to the control, whereas treatment with Salvia miltiorrhiza dose dependently suppressed cellular ROS level induced by Ang II. Among them, Salvia miltiorrhiza at the highest concentration has the most potent antioxidative effect on mesangial cells. Salvia miltiorrhiza alone did not alter the basal cellular ROS (Fig. 3).

**Effect of Salvia miltiorrhiza on TGF-β1 Release from Mesangial Cells Induced by Ang II**

TGF-β1 in conditioned media was quantitatively measured by ELISA method. When mesangial cells were incubated in the presence of 100 nM Ang II for 24 hours, the concentration...
Figure 3. Effects of Salvia miltiorrhiza injection in the presence or absence of Ang II on reactive oxygen species in mesangial cells. (A) Representative image of fluorescent signals generated from composite images obtained by confocal microscopy of mesangial cells pretreated with the radical probe CM-H$_2$DCFDA for 45 min. A: Control; B: mesangial cells + Ang II; C: mesangial cells + Ang II + SM (37.5 mg/ml); D: mesangial cells + Ang II + SM (75 mg/ml); E: mesangial cells + Ang II + SM (150 mg/ml); F: mesangial cells + SM (150 mg/ml). (B) Results of cellular ROS analyzed by Leica Confocal Software program are graphically shown. Lane 1: untreated control; lane 2: treatment with 100 nM Ang II; lane 3: treatment with Ang II + SM (37.5 mg/ml); lane 4: treatment with Ang II + SM (75 mg/ml); lane 5: treatment with Ang II + SM (150 mg/ml); lane 6: treatment with SM (150 mg/ml); * $p < 0.05$ vs. the control; ** $p < 0.01$ vs. the control; ## $p < 0.01$ vs. the group treated by Ang II.
of TGF-β₁ in the conditioned medium increased from 75.11 ng/ml ± 23.37 ng/ml to 151.15 ng/ml ± 27.59 ng/ml. After mesangial cells were pretreated with 37.5 mg/ml, 75 mg/ml, and 150 mg/ml Salvia miltiorrhiza respectively, the concentration of TGF-β₁ in the conditioned medium was significantly decreased. The decrease of TGF-β₁ level was in a concentration dependent manner. Salvia miltiorrhiza alone did not inhibit basal level of TGF-β₁ protein secretion (Fig. 4).

Discussion

Salvia miltiorrhiza, a commonly used herb, has many effects. The previous investigations of Salvia miltiorrhiza were frequently confined to the treatment of atherosclerosis-related disorders. Its effects on chronic hepatic diseases, such as liver fibrosis and cirrhosis, have been proven in experiments in vitro (Chor et al., 2005) and in vivo (Lee et al., 2003). In the present study, we demonstrated that Ang II can increase PAI-1 mRNA and protein expression in cultured mesangial cells which have been shown to be a key regulator of glomerular sclerosis. We also demonstrated that Salvia miltiorrhiza can decrease Ang II-induced PAI-1 mRNA and protein expression. At the same time, Salvia miltiorrhiza effectively inhibited TGF-β₁ secretion and cellular ROS induced by Ang II as well.

The renin-angiotensin system (RAS) plays an important role in the progression of renal disease. RAS causes renal injury and fibrosis, in part, through effects on PAI-1 (Chor et al., 2005). Our study also indicated that Ang II can increase PAI-1 secretion in mesangial cells. But Salvia miltiorrhiza can decrease Ang II-induced PAI-1 mRNA expression and protein synthesis in mesangial cells which have been shown to be key regulators of matrix
accumulation and glomerulosclerosis. Furthermore, we observed a dose-dependent decrease in PAI-1 mRNA expression and protein secretion in the presence of Salvia miltiorrhiza.

Ang II also induced an increase in the expression of TGF-β1 in cultured mesangial cells in this study, which was in good agreement with results from previous study (Jaimes et al., 2005). Substantial clinical and experimental evidences suggest that TGF-β1 is the key mediator of glomerular fibrosis (Ziyadeh et al., 1998; Peters et al., 1999). The powerful fibrogenic action of Ang II acts mainly through stimulating production of TGF-β1 (Lee et al., 2003). TGF-β1 promotes glomerular fibrosis not only by enhancing ECM synthesis, but also by decreasing ECM degradation. TGF-β1 degrades and turnovers the ECM mainly through stimulating the synthesis of PAI-1 in mesangial cells (Baricos et al., 1999) and PAI-1 gene deficiency can attenuate TGF-β1-induced interstitial ECM deposition (Krag et al., 2005). TGF-β1 is a potent inducer of PAI-1 gene expression (Dennler et al., 1998).

In this study, Ang II increased cellular ROS in mesangial cells as well. It has long been recognized that ROS are harmful for cells, mainly because they injure lipids, proteins, and nucleic acids, which leads to structural and functional impairments (Rodrigo and Bosco, 2006). The glomerulus is considerably more sensitive to oxidative injuries than other nephron segments. Oxidative stress may alter the structure and function of the glomerulus because of the effect of ROS on mesangial cells (Rodrigo and Bosco, 2006). Previous studies have shown that Ang II induces mesangial cell hypertrophy via ROS (Lin et al., 2006; Wojcikowski et al., 2006). ROS has close relationship with up-regulation of PAI-1 expression too (Lee et al., 2005). The promoter region of PAI-1 gene contains binding sites for several redox sensitive transcription factors such as Sp1, AP-1, and NF-κB (Rerolle et al., 2000). Many experiments have shown that Salvia miltiorrhiza can protect cells against oxidative stress (Lin et al., 2006; Zhang and Wang, 2006).

We observed dose-dependent decreases in ROS production and TGF-β1 protein expression in the presence of Salvia miltiorrhiza. Perhaps, the down-regulation of TGF-β1 expression and cellular ROS level in mesangial cells have some correlation with the decrease of PAI-1 mRNA and protein secretion after being treated by Salvia miltiorrhiza.

In conclusion, the results of this study demonstrated that Salvia miltiorrhiza can decrease cellular ROS level and TGF-β1 and PAI-1 secretion in mesangial cells, which due to its ability of inhibiting the activities of angiotensin II. Therefore, Salvia miltiorrhiza can be used to ameliorate progression of glomerular sclerosis.

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References


