# Matrine inhibits the expression of adhesion molecules in activated vascular smooth muscle cells

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Abstract. Atherosclerosis is a chronic inflammatory disease associated with increased expression of adhesion molecules in vascular smooth muscle cells (VSMCs). Matrine is a main active ingredient of Sophora flavescens roots, which are used to treat inflammatory diseases. However, the effects of matrine on the expression of adhesion molecules in VSMCs have largely remained elusive. Therefore, the present study investigated the effects of matrine on the expression of adhesion molecules in tumor necrosis factor (TNF)-a-stimulated human aortic smooth muscle cells (HASMCs). The results showed that matrine inhibited the expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) in TNF- $\alpha$ -stimulated HASMCs. Matrine markedly inhibited the TNF-a-induced expression of nuclear factor (NF)-kB p65 and prevented the TNF- $\alpha$ -caused degradation of inhibitor of NF- $\kappa$ B; it also inhibited TNF-a-induced activation of mitogen-activated protein kinases (MAPKs). Furthermore, matrine inhibited the production of intracellular reactive oxygen species (ROS) in TNF-a-stimulated HASMCs. In conclusion, the results of the present study demonstrated that matrine inhibited the expression of VCAM-1 and ICAM-1 in TNF-α-stimulated HASMCs via the suppression of ROS production as well as NF-kB and MAPK pathway activation. Therefore, matrine may have a potential therapeutic use for preventing the advancement of atherosclerotic lesions.

## Introduction

Atherosclerosis is now recognized as a chronic inflammatory disease of the vascular wall (1). Cell-cell and cell-matrix adhesion have important roles in the formation of atherosclerotic

lesions (2). As the disease progresses, vascular smooth muscle cells (VSMCs) undergo phenotypic transformation and become activated to secrete pro-inflammatory cytokines and monocyte chemoattractant protein 1, and express cell adhesion molecules that promote leukocyte recruitment, migration and differentiation (3). Accumulating evidence implied that vascular cell adhesion molecule-1 (VCAM-1) is upregulated in VSMCs of atherosclerotic lesions (4-6). Furthermore, in cultured VSMCs, interleukin-1 (IL-1) and tumor necrosis factor (TNF- $\alpha$ ) induced VCAM-1 and intercellular adhesion molecule-1 (ICAM-1) expression as well as monocyte adhesion to VSMCs (7,8). Therefore, preventing the expression of these adhesion molecules on VSMCs may be a promising therapeutic approach for atherosclerosis.

Compelling evidence has revealed that certain natural products, particularly those from medicinal plants, may represent an ideal source to develop safe and effective agents for the management of atherosclerosis. Stereocalpin A, an active component of the Antarctic lichen *Ramalina terebarata*, prevented the induction of the expression of adhesion molecules in a concentration-dependent manner after stimulation with an inflammatory cytokine (9). Sulforaphane, a compound naturally occurring in *Brassica oleracea* var. italica (broccoli) and numerous other cruciferous vegetables, was also shown to inhibit the expression of TNF- $\alpha$ -induced adhesion molecules in VSMCs (10).

Matrine is a major active component of Sophora flavescens roots, which are used to treat inflammatory diseases, including enteritis and hepatitis (11,12). Besides its anti-inflammatory activity, matrine has been shown to affect the cardiovascular system. A previous study reported that matrine inhibits VSMC proliferation via upregulation of the p53/p21 signaling pathway (13). However, the effects of matrine on the expression of adhesion molecules in VSMCs have remained elusive. The purpose of the present study was to investigate the effects of matrine on adhesion molecule accumulation in TNF-a-stimulated human aortic smooth muscle cells (HASMCs) as well as the underlying mechanisms of action. The results demonstrated that matrine suppressed TNF-a-induced adhesion molecule expression through the inhibition of mitogen-activated protein kinase (MAPK) and nuclear factor (NF)-kB signaling pathways and intracellular reactive oxygen species (ROS) production in HASMCs.

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#### Materials and methods

Materials. Matrine (purity, >99%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Dulbecco's modified Eagle's medium, fetal bovine serum (FBS) and Lipofectamine Plus were purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The pGL3-NF- $\kappa$ B vector and the luciferase assay system were provided by Promega (Madison, WI, USA), and the pCMV-β-gal vector was obtained from Lonza (Walkersville, MD, USA). Antibodies against ICAM-1 (mouse monoclonal) and VCAM-1 (mouse monoclonal) were purchased from R&D Systems, Inc. (Minneapolis, MN, USA) and antibodies against the inhibitor of NF- $\kappa$ B (I $\kappa$ B- $\alpha$ ; rabbit polyclonal), p65 (rabbit polyclonal), c-Jun N-terminal kinase (JNK; rabbit polyclonal), phospho-JNK (p-JNK; rabbit polyclonal), extra-cellular signal-regulated kinase (ERK; rabbit monoclonal), p-ERK (rabbit polyclonal), p38 (rabbit polyclonal), p-p38 (rabbit polyclonal), Akt, p-Akt, lamin A (rabbit polyclonal) and  $\beta$ -actin (rabbit polyclonal) were purchased from Abcam Inc. (Cambridge, MA, USA). PCR primers and PCR premix were purchased from Bioneer Corporation (Daejeon, South Korea). phosphate-buffered saline (PBS), tris-buffered saline (TBS) and Tween 20 were purchased from Sigma-Aldrich. (St. Louis, MO, USA).

*Cell culture*. HASMCs were purchased from Clonetics Corp. (San Diego, CA, USA) and cultured in DMEM medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 100 IU/ml penicillin, 100 mg/ml streptomycin, and 5% FBS (Invitrogen; Thermo Fisher Scientific, Inc.) with 2 ng/ml basic fibroblast growth factor, 10 ng/ml recombinant human epidermal growth factor and 5  $\mu$ g/ml insulin (all from Sigma-Aldrich).

*Cell viability assay.* The effects of matrine on the proliferation of HASMCs were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HASMCs were seeded onto 96-well plates (1x10<sup>4</sup> cells/well) and subsequently treated with various concentrations of matrine (0, 10, 50 and 100  $\mu$ g/ml). Following 72 h of incubation, MTT (0.5 mg/ml; Sigma-Aldrich) was added to each well. After incubation for 4 h, the supernatant was removed and 0.1% dimethylsulfoxide (Sigma-Aldrich) was added to dissolve the formazan crystals. The absorbance at 550 nm was measured using a microplate reader 3350 (Bio-Rad Laboratories, Hercules, CA, USA) and the percentage of viable cells compared with that in the control group was calculated.

Reverse-transcription quantitative polymerase chain reaction (RT-qPCR). RNA extraction was performed with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Subsequently, RNA was reverse-transcribed in a 20-ml reaction system using an Advantage RT kit (Clontech Laboratories, Inc., Palo Alto, CA, USA) according to the supplier's recommended protocol. mRNA levels were quantified by RT-qPCR using SYBRGreen Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) using a 30 ng template in a 20  $\mu$ l reaction mixture. For PCR amplification, the following primers were used: VCAM-1 forward, 5'-CAAAGGTGG



Figure 1. Effect of matrine on HAMSC viability. HAMSCs were treated with various concentrations (0, 10, 50 and 100  $\mu$ g/ml) of matrine for 24 h. The cytotoxicity was then measured by an MTT assay. All experiments were repeated at least three times. Values are expressed as the mean  $\pm$  standard error of the mean. HAMSC, human aortic smooth muscle cell.

ATCAGATTCAAG-3' and reverse, 5;-GGTGAGCATTAT CACCCAGAA-3'; ICAM-1 forward, 5'-CAAAGGTGG ATCAGATTCAAG-3' and reverse, 5;-GGTGAGCATTAT CACCCAGAA-3'; GAPDH forward, 5'-CAAAGGTGG ATCAGATTCAAG-3' and reverse, 5;-GGTGAGCATTAT CACCCAGAA-3'. The PCR cycling program was 95°C for 3 min, followed by 28 cycles of 95°C for 20 sec, 60°C for 20 sec and 72°C for 15 sec, and a final extension at 72°C for 5 min. The levels of individual gene mRNA transcripts were initially normalized to the control  $\beta$ -actin. Subsequently, the differential expression of these genes was analyzed using the 2- $\Delta\Delta$ Cq method (14).

Western blot analysis. HVSMCs were cultured and treated with matrine as described above and subsequently subjected to western blot analysis. After treatment, the cells were washed twice with PBS and suspended in 70  $\mu$ l of Buffer A [10 mM HEPES (pH 7.9), 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF and Protease Inhibitor Cocktail (Sigma-Aldrich)] and incubated on ice. After 15 min, 0.5% Nonidet P (NP)-40 was added to lyse the cells, which were vortexed for 1 sec. Then, cytosolic cell extracts were obtained after centrifuging at 1500 x g for 10 min at 4°C. The collected nuclei were resuspended in 50  $\mu$ l of Buffer C [20 mM HEPES (pH 7.9), 1.5 mM MgCl<sub>2</sub>, 420 mM NaCl, 0.2 mM EDTA, 25% v/v glycerol, 0.5 mM PMSF and Protease Inhibitor Cocktail] and incubated on ice for 20 min with intermittent agitation. Nuclear cell extracts were recovered after centrifugation for 10 min at 13,000 x g at 4°C. The protein concentration in the cell extracts was then determined using the Bradford protein dye reagent (Bio-Rad Laboratories, Inc.). Proteins (30 µg/lane) were separated on 10% sodium dodecyl sulfate polyacrylamide gels (Bio-Rad Laboratories) and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 5% non-fat milk in TBST buffer (50 mM Tris, pH 7.5, 250 mM NaCl, 0.1% Tween 20) and incubated with the appropriate primary antibodies overnight at 4°C. Subsequent to washing the membranes three times with phosphate-buffered saline, 5 min per wash, containing 0.1% (v/v) Tween 20, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (MyBioSource, Inc., San Diego, CA, USA) for 1 h, followed



Figure 2. Effect of matrine on adhesion molecule expression in HAMSCs. HAMSCs were pre-incubated with various concentrations (0, 10, 50 and 100  $\mu$ g/ml) of matrine for 2 h and stimulated with TNF- $\alpha$  (10 ng/ml) for 4 h. mRNA expression of (A) VCAM-1 and (B) ICAM-1 was determined by reverse-transcription quantitative polymerase chain reaction analysis. (C) Protein expression of VCAM-1 and ICAM-1 was determined by western blot analysis. All experiments were repeated at least three times. Values are expressed as the mean ± standard error of the mean. \*P<0.05 vs. TNF- $\alpha$  group. HAMSC, human aortic smooth muscle cell. TNF, tumor necrosis factor; VCAM, vascular cell-adhesion molecule; ICAM, intercellular cell-adhesion molecule.

by visualization of the antibodies using enhanced chemiluminescence detection reagents. The blots were developed using an enhanced chemiluminescence kit (GE Healthcare Life Sciences, Chalfont, UK). Semi-quantitative determination of protein levels was performed using Image-Pro Plus software (NIH Image J 1.61; Media Cybernetics, Rockville, MD, USA).

*Transfection and reporter assays.* The cells (1x10<sup>5</sup> cells/ml) were plated into each well of a 6-well plate. The cells were transiently co-transfected with the plasmids, pGL3-NF-κB and pCMV-β-gal using Lipofectamine Plus according to the manufacturer's protocol. Briefly, a transfection mixture containing 0.5  $\mu$ g pGL3-NF-κB and 0.2  $\mu$ g pCMV-β-gal was mixed with the Lipofectamine Plus reagent and added to the cells. After 4 h, the cells were pretreated with matrine, and then lysed with 200  $\mu$ l of lysis buffer (24 mM Tris-HCl (pH 7.8), 2 mM dithiotreitol, 2 mM EDTA, 10% glycerol, and 1% Triton X-100) and 10  $\mu$ l of cell lysates were used for luciferase activity assay. The luciferase and β-galactosidase activities were determined. The

values shown represent an average of three independent transfections, which were normalized with  $\beta$ -galactosidase activity. Each transfection was performed in triplicate and experiments were repeated three times.

Assessment of ROS levels. ROS levels were determined according to the method of a previous study (15). 5,6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CMH<sub>2</sub>DCFDA; Molecular Probes, Eugene, OR, USA) was used to determined intracellular ROS levels using flow cytometry. Following pre-treatment of HASMCs (3x10<sup>6</sup> cells/ml) with various concentrations of matrine for 2 h, cells were incubated with TNF- $\alpha$  (10 ng/ml) for 4 h. The cells were then stained with 5  $\mu$ M CMH<sub>2</sub>-DCFDA for 15 min at 37°C. The cells were kept in the dark on ice and at least 10,000 cells for each sample were analyzed using a Becton Dickinson FACSCalibur (BD Biosciences, San Jose, CA, USA). Changes in the levels of intracellular ROS are expressed as a percentage of TNF- $\alpha$ -stimulated, matrine-untreated cells.



Figure 3. Effects of matrine on NF- $\kappa$ B activation and I $\kappa$ B $\alpha$  degradation in TNF- $\alpha$ -stimulated HASMCs. (A) HAMSCs were transfected with a pGL3-NF- $\kappa$ B-Luc reporter plasmid and pCMV- $\beta$ -gal, pre-treated with various concentrations of matrine (0, 10, 50 and 100  $\mu$ g/ml) for 2 h and stimulated with TNF- $\alpha$  for 4 h. Values are expressed as the mean  $\pm$  standard error of the mean. \*P<0.05 vs. TNF- $\alpha$  group. (B) HAMSCs were pre-incubated with or without various concentrations of matrine for 2 h and then treated with TNF- $\alpha$  for 4 h. Cytoplasmic and nuclear levels of NF- $\kappa$ B p65 were detected by western blot analysis to determine the translocation of NF- $\kappa$ B. Lamin A and  $\beta$ -actin were used as loading controls for nuclear and cytosolic protein fractions, respectively. (C) I $\kappa$ B $\alpha$  degradation was analyzed by western blot analysis. All experiments were repeated at least three times. HAMSC, human aortic smooth muscle cell; NF- $\kappa$ B, nuclear factor kappa B; I $\kappa$ B $\alpha$ , inhibitor of NF- $\kappa$ B; TNF, tumor necrosis factor.

Statistical analysis. Values are expressed as the mean  $\pm$  standard error of the mean. Data from different groups were compared using a Student's *t*-test or one-way analysis of variance followed by Dunnett's test. Statistical analysis was performed using SPSS (version 12.5S; SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

### Results

*Matrine does not affect HAMSC viability.* To examine the effect of matrine on cell viability, HASMCs were treated with various concentrations of matrine for 8 h and subjected to an MTT assay. As shown in Fig. 1, no significant cytotoxicity of matrine was observed. These observations indicated that matrine had no effect on the viability of HASMCs.

Matrine inhibits TNF- $\alpha$ -mediated induction of adhesion molecules in HAMSCs. To examine whether matrine affects TNF- $\alpha$ -mediated induction of adhesion molecules, HASMCs were pre-treated with various concentrations of matrine for 2 h, followed by stimulation with TNF- $\alpha$  (10 ng/ml) and subjected to RT-qPCR analysis. As shown in Fig. 2A and B, treatment with TNF- $\alpha$  induced the mRNA expression of VCAM-1 and ICAM-1 on HASMCs. However, matrine significantly inhibited TNF- $\alpha$ -induced mRNA expression of VCAM-1 and ICAM-1 in a concentration-dependent manner. Consistent with these results, western blot analysis showed that matrine obviously suppressed TNF- $\alpha$ -induced protein expression of VCAM-1 and ICAM-1 in a concentration-dependent manner (Fig. 2C). These results suggested that matrine effectively blocks TNF- $\alpha$ -induced expression of VCAM-1 and ICAM-1. Matrine inhibits TNF- $\alpha$ -induced NF- $\kappa B$  activation in HAMSCs. Activation of NF-kB is linked with the development of vascular damage; furthermore, transcription factors are known to mediate the expression of adhesion molecules (16). Therefore the present study examined the effects of matrine on NF- $\kappa$ B-mediated transcriptional activation. HASMCs were treated with various concentrations of matrine for 2 h and subsequently stimulated with TNF- $\alpha$  for 4 h. Transcriptional activation assays were then employed to determine whether matrine affects NF-kB-dependent transcription. Stimulation with TNF- $\alpha$  obviously increased luciferase activity, while matrine significantly prevented this effect (Fig. 3A). Furthermore, the expression of NF-KB p65 protein was detected by western blot analysis to clarify the inhibitory action of matrine. As shown in Fig. 3B, pre-treatment of HASMCs with matrine significantly decreased the nuclear levels of NF-KB p65, while simultaneously increasing its cytosolic levels. Furthermore, the effects of matrine on IkB protein in TNF- $\alpha$ -stimulated HASMCs were determined. TNF- $\alpha$ caused a significant degradation of IkBa at 30 min, which was inhibited by matrine (Fig. 3C). These results suggested that matrine impedes TNF-α-induced NF-κB activation.

Matrine inhibits TNF- $\alpha$ -induced MAPK activation in HAMSCs. MAPK signaling is involved in the regulation of adhesion-molecule expression (16). Therefore, the present study investigated the effects of matrine on TNF- $\alpha$ -induced phosphorylation of MAPKs in HAMSCs. As shown in Fig. 4, TNF- $\alpha$  significantly increased the activation of p38/MAPK, ERK1/2 and JNK in matrine-untreated cells. However, matrine concentration-dependently inhibited TNF- $\alpha$ -induced phosphorylation of MAPKs in HAMSCs.



Figure 4. Effects of matrine on TNF- $\alpha$ -induced phosphorylation of MAPKs in HAMSCs. HAMSCs were pre-treated with various concentrations of matrine (0, 10, 50 and 100 µg/ml) for 2 h and then incubated with TNF- $\alpha$  (10 ng/ml) for 30 min. The relative intensities were expressed as the ratio of phospho-MAPK to total MAPK. Data were normalized to  $\beta$ -actin and are expressed as fold-changes of  $\beta$ -actin. All experiments were repeated at least three times. Values are expressed as the mean ± standard error of the mean. \*P<0.05 vs. TNF- $\alpha$  group. HAMSC, human aortic smooth muscle cell; MAPK, mitogen-activated protein kinase; p-ERK, phosphorylated extracellular signal-regulated kinase; TNF, tumor necrosis factor; JNK, c-Jun N-terminal kinase.

Matrine reduces ROS production in TNF- $\alpha$ -stimulated HAMSCs. As it has been reported that TNF- $\alpha$ -induced ROS production activates NF- $\kappa$ B in vascular cells, the present study investigated the effect of matrine on the production of TNF- $\alpha$ -induced ROS production in HAMSCs (17). HAMSCs were pre-treated with matrine for 2 h and then stimulated with TNF- $\alpha$ . As shown in Fig. 5, matrine significantly reduced the production of TNF- $\alpha$ -induced ROS in a concentration-dependent manner. The production of ROS was reduced to ~50% by the highest concentration of matrine (100  $\mu$ g/ml).

#### Discussion

Cytokines such as IL-1 and TNF- $\alpha$  have been shown to induce the expression of cellular adhesion molecules VCAM-1 and ICAM-1 in atherosclerosis (18). VSMCs express VCAM-1 and ICAM-1, which are prominent in the fibrous caps of advanced atherosclerotic plaques (19). Therefore, pharmacological agents that inhibit the expression of these adhesion molecules have are potential drugs for inhibiting atherosclerosis. The present study showed that matrine inhibits the expression of VCAM-1 and ICAM-1 in TNF- $\alpha$ -stimulated HASMCs via the suppression of ROS production as well as NF- $\kappa$ B and MAPK pathway activation.



Figure 5. Effects of matrine on the production of ROS in TNF- $\alpha$ -stimulated HAMSCs. HAMSCs were treated with various concentrations of matrine (0, 10, 50 and 100 µg/ml) for 2 h followed by stimulation with TNF- $\alpha$  (10 ng/ml) for 4 h and determination of ROS levels. All experiments were repeated at least three times. Values are expressed as the mean ± standard error of the mean. \*P<0.05 vs. TNF- $\alpha$  group. HAMSC, human aortic smooth muscle cell; ROS, reactive oxygen species; TNF, tumor necrosis factor.

A previous study showed that the expression of adhesion molecules, including VCAM-1 and ICAM-1, is increased in coronary atheroscleorotic tissue (20). In addition, inflammatory cytokines, including IL-1 $\beta$  and TNF- $\alpha$ , increase the expression of VCAM-1 and ICAM-1 (21,22). The present study demonstrated that TNF- $\alpha$  significantly upregulated VCAM-1 and ICAM-1 expression in HAMSCs. These results were consistent with those of earlier published studies. However, matrine prevented the TNF- $\alpha$ -induced expression of VCAM-1 and ICAM-1. The present study showed that matrine has inhibitory effects on adhesion molecule expression in HASMCs stimulated with TNF- $\alpha$ .

In all vascular cells implicated in the development of atherosclerosis, inflammatory mediators stimulate NF-KB activation (23-25). In quiescent cells, due to its association with  $I\kappa B$ , NF- $\kappa B$  is localized to the cytoplasm and unable to translocate to the nucleus (26). However, IkB is phosphorylated, ubiquitinated and subsequently degraded via the proteasome pathway in lipopolysaccharide- and cytokine-activated cells, which facilitates the nuclear translocation of NF- $\kappa$ B, where it initiates the transcription of numerous genes, including pro-inflammatory cytokines, cell adhesion molecules and chemokines (27,28). The results of the present study demonstrated that matrine decreased TNF-α-induced NF-kB activation through inhibition of IkB kinase activation and subsequent IkBa degradation. Collectively, these results revealed that the inhibitory effects of matrine on the expression of adhesion molecules is, at least partially, mediated through the suppression of NF-κB activation.

In addition to NF- $\kappa$ B, the MAPK signaling pathway also has a major role in diseases associated with vascular remodeling, as it regulates cell adhesion, proliferation, apoptosis and migration (29-31). TNF- $\alpha$ -activated signaling pathways may also include MAPKs, which may mediate the resulting inflammatory responses (32). To better identify the effects of matrine on the MAPK signaling pathway, the present study assessed its impact on the levels of total and phosphorylated p38/MAPK, JNK and ERK1/2 in TNF- $\alpha$ -treated HASMCs. The results revealed that the TNF- $\alpha$ -induced phosphorylation of these MAPKs was significantly reduced by matrine in a concentration-dependent manner. Therefore, it was indicated that matrine downregulated adhesion molecule expression induced with TNF- $\alpha$  through inhibition of MAPK activation.

ROS serve as secondary messengers, which activate multiple signaling pathways, including NF- $\kappa$ B and MAPKs, leading to the induction of numerous downstream genes with essential roles in the physiology and pathophysiology of vascular cells (27,33). In addition, the expression of adhesion molecules has been shown to be stimulated via the NF- $\kappa$ B signaling pathway, which was activated by induction of ROS (34). The results of the present study demonstrated that matrine significantly and concentration-dependently decreased the ROS production induced by TNF- $\alpha$ . These findings indicated that in TNF- $\alpha$ -treated HASMCs, matrine inhibits the activation of NF- $\kappa$ B and MAPKs via suppressing ROS production.

In conclusion, the results of the present study suggested that matrine reduced the expression of VCAM-1 and ICAM-1 in HASMCs stimulated with TNF- $\alpha$  via the suppression of ROS production and consequently of NF- $\kappa$ B and MAPK pathway activation. Therefore, matrine was indicated to be an effective anti-inflammatory agent with potential therapeutic use for preventing the advancement of atherosclerotic lesions.

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