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Stereocalpin A inhibits the expression of adhesion molecules in activated vascular smooth muscle cells

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ABSTRACT

Up-regulation of cell adhesion molecules on vascular smooth muscle cells (VSMCs) and leukocyte recruitment to the vascular wall contribute to vascular inflammation and atherosclerosis. Stereocalpin A, a chemical compound of the Antarctic lichen *Ramalina terebarata*, displays tumoricidal activity against several different tumor cell types. However, other biological activities of stereocalpin A and its molecular mechanisms remain unknown. In this study, our work is directed toward studying the in vitro effects of stereocalpin A on the ability to suppress the expression of adhesion molecules induced by TNF-α in vascular smooth muscle cells. Pretreatment of VSMCs for 2 h with stereocalpin A at nontoxic concentrations of 0.1–10 μg/ml inhibited TNF-α-induced adhesion of THP-1 monocytic cells and expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1). Stereocalpin A reduced TNF-α-induced production of intracellular reactive oxygen species (ROS) and phosphorylation of p38, ERK, JNK and Akt. Stereocalpin A also inhibited NK-κB activation induced by TNF-α. Moreover, stereocalpin A inhibited TNF-α-induced IκB kinase activation, subsequent degradation of IκBα, and nuclear translocation of NF-κB. Hence, we describe a new anti-inflammatory activity and mechanism of stereocalpin A, owing to the negative regulation of TNF-α-induced adhesion molecule and MCP-1 expression, monocyte adhesion and ROS production in vascular smooth muscle cells. These results suggest that stereocalpin A has the potential to exert a protective effect by modulating inflammation within the atherosclerotic lesion.

1. Introduction

The onset of atherosclerosis is marked by an inflammatory response, characterized by the accumulation of lipids and fibrous elements in the vessel wall of arteries, which is the main cause of cardiovascular disease. Previous studies have mentioned an important role for vascular smooth muscle cell (VSMCs) in the initiation of atherosclerosis [1–3]. As the disease progresses, VSMCs are in proximity to and physically interact with inflammatory leukocytes, which perform a very important function in further exacerbating the disease [4]. This process is mainly due to enhanced expression of adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1). The expression of these molecules has been detected on intimal VSMCs and may facilitate the accumulation of transmigrated leukocytes within the atherosclerotic vascular wall [5,6]. In addition, adhesion molecule expression is influenced by the cytokine milieu including TNF-α in vascular cells [7]. Therefore, a promising therapeutic approach for treating pathological inflammation is to reduce aberrant leukocyte adhesion to the VSMCs via suppression of adhesion molecule expression.

There has been increasing interest in the flora and fauna of the Antarctic in recent years. In the Antarctic region lichens form the main part of the flora and many lichen species are found [8]. Lichens represent a source of natural products formed by symbiotic associations between heterotrophic fungi and cyanobacteria or algae. Lichens and their natural products have been used for cosmetics, food and natural remedies [9,10]. In addition, they exert a broad range of important biological actions comprising antibiotic, antimycobacterial, antiviral, antiinflammatory, analgesic, antipyretic, antiproliferative and cytotoxic effects [11]. However, there is still large potential for further industrial screening and research on lichen products and their therapeutic potential remains pharmaceutically unexploited.

Stereocalpin A has been isolated from the MeOH extract of the Antarctic lichen *Ramalina terebarata* [12]. Stereocalpin A is a unique cyclic peptide incorporating an unprecedented 5-hydroxy-2,4-dimethyl-3-oxo-octanoic acid in the structure (Fig. 1). It has been suggested that stereocalpin A has a tumoricidal activity against several different tumor cell types. However, published scientific information regarding

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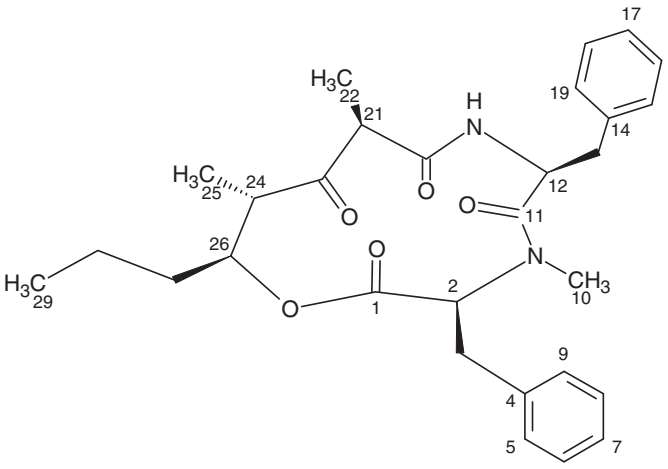

Fig. 1. Chemical structure of stereocalpin A.

other biological activities of stereocalpin A and its molecular mechanisms is scarce.

The aim of this study was to investigate the effects and mechanisms of action of stereocalpin A in the context of adhesion molecule accumulation induced by TNF-α. The present study suggests that stereocalpin A can inhibit TNF-α-induced adhesion molecule and MCP-1 expression through the inhibition of MAPK, Akt and NF-κB signaling pathways and intracellular ROS production in vascular smooth muscle cells.

2. Materials and methods

2.1. Reagents

Unless otherwise indicated, all the chemicals used in this study were purchased from Sigma Chemical Co. (St Louis, MO). Anti-ICAM-1 and anti-VCAM-1 antibodies were purchased from R & D Systems, USA. Lipofetamine Plus, DMEM medium and fetal bovine serum were purchased from Life Technologies, Inc. (Carlsbad, CA). pGL3-NF-κB and the luciferase assay system were obtained from Promega (Madison, WI). pCMV-β-gal was obtained from Lonza (Walkersville, MD). 3-amino-1,2,4-triazole was purchased from Calbiochem (La Jolla, CA). Antibodies against IκB-α, p65, JNKs, phospho-JNK (p-JNK), ERK, phopho-ERK (p-ERK), phospho-38, p38, phosphor-Akt, Akt, lamin A and β-actin were purchased from Abcam Inc., USA. Stereocalpin A was kindly provided by Dr. J. Yim (Polar BioCenter, Korea Polar Research Institute).

2.2. Cell culture

The vascular smooth muscle cell line MOVAS-1, was purchased from ATCC (Rockville, MD). The cells were grown in DMEM meidum supplemented with 200 mg/ml G418, 100 IU/ml penicillin, 100 mg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (FBS) in a humidified atmosphere containing 5% CO_2 incubator at 37 °C. For subculturing, the cells were detached using 0.125% trypsin containing 0.01 M EDTA. Cells used in this study were from the first to sixth passage. All experiments were carried out with the same batch of MOVAS-1, which were from single donor. Human aortic smooth muscle cells (HASMC) and human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics Corp. (San Diego, CA). HASMC were cultured in MCDB 131 medium supplemented with 5% FBS, 10 ng/ml recombinant human epidermal growth factor, 2 ng/ml basic fibroblast growth factor, and 5 μg/ml insulin. HUVEC were grown in EGM-2 medium in gelatin coated tissue culture flasks in a humidified atmosphere containing 5% CO_2 incubator at 37 °C. For subculturing, the cells were detached using 0.125% trypsin containing 0.01 M EDTA. Cells were passaged every 3–5 days, and experiments were performed on cells five to eight passages from primary culture. THP-1 cells (ATCC), a human myelomonocytic cell line, are widely used to study monocyte/macrophage biology in culture systems [13]. THP-1 was used for our cell adhesion assay with MOVAS-1. These cells were cultured in RPMI 1640, and supplemented with 2 mM L-glutamine, 100 μg/ml streptomycin, 100 IU/ml penicillin, and 10% FBS.

2.3. Cell adhesion assay

The methodology used for the adhesion assays is described elsewhere [14]. Briefly, VSMCs, which were grown in 96 well plates, were pre-treated with stereocalpin A (0.1–10 μg/ml) for 2 h. The cells were washed with medium to remove stereocalpin A and incubated with fresh growth medium containing TNF-α (10 ng/ml) for 6 h. The medium was removed from the wells and BCECF-labeled THP-1 cells (2.5×10^5 cells/ml) in 0.2 ml of the medium were added to each well. The test and control samples were performed in triplicates in each experiment. After incubation for 1 h at 37 °C in 5% CO_2, the microwells were washed twice with 0.2 ml of warm medium and a number of adherent cells were determined by measuring the fluorescence intensity using a Cytoflour 2350 (Millipore, Bedford, MA). Increase in THP-1 cells adhesion upon stimulation of MOVAS-1 with TNF-α was calculated in relation to the basal adhesion of THP-1 cells to non-stimulated VSMCs that was set to 100.

2.4. Determination of cell surface expression of adhesion molecules by ELISA

The cell surface expression of the adhesion molecules on the muscle cell monolayers was quantified by ELISA using a modification of the methods described previously [15]. VSMCs were seeded at a concentration of 2×10^4 cells/well in 96-well gelatin-coated plates, cultured to confluence and pre-treated with stereocalpin A (0.1–10 μg/ml) for 2 h at 37 °C. These pre-treated cells were washed with medium to remove stereocalpin A and then incubated with fresh growth medium containing TNF-α (10 ng/ml) for 8 h for measurement of VCAM-1 and ICAM-1. After incubation, the cells were washed with phosphate buffer saline pH 7.4 (PBS) and fixed with 1.0% glutaraldehyde for 30 min at 4 °C. Bovine serum albumin (1.0% in PBS) was added to the cells to reduce non-specific binding. The cells were then incubated with monoclonal antibodies against either ICAM-1, VCAM-1, or isotype matched control antibody (0.25 g/ml, diluted in blocking buffer) overnight at 4 °C, washed with PBS and incubated with alkaline phosphatase-conjugated goat anti-mouse secondary antibody (1 μg/ml, diluted in PBS). The cells were then washed with PBS and exposed to the peroxidase substrate (p-nitorphenyl phosphate 1 mg/ml in 0.1 M glycin buffer, pH 10.4 containing 1 mM $MgCl_2$, and 1 mM $ZnCl_2$). The absorbance was measured at 405 nm using a Molecular device microplate reader (Menlo Park, CA). The absorbance values of the isotype matched control antibody were taken as the blank, which were subtracted from the experimental values.

2.5. Measurement of mRNA levels by reverse transcription polymerase chain reaction (RT-PCR)

The total RNA was extracted using a single-step guanidinium thiocyanate-phenol-chloroform method. The yield and purity of the RNA were confirmed by measuring the ratio of the absorbances at 260 and 280 nm. PCR was performed using ICAM-1-, or VCAM-1-specific primers to identify their respective specific cDNA. The following mouse ICAM-1-specific primers were synthesized: sense primer, 5′-CCTGTTTCCTGCCTCTGAAG-3′ and antisense primer, 5′-GTCTGCTGAGACCCCTCTTG-3′. The following

mouse VCAM-1-specific primers were synthesized: sense primer, 5′-CCCAAGGATCCAGAGATTCA-3′ and antisense primer, 5′-TAAGGTGAGGGTGGCATTTC-3′. Mouse GAPDH PCR primers were 5′-GGTCCTCAGTGTAGCCCAAG-3′ (sense) and 5′-AATGTGTCCGTCGTG-GATCT-3′ (antisense). The following human ICAM-1-specific primers were synthesized: sense primer, 5′-CTCACCGTGTACTG-GACTCC-3′ and antisense primer, 5′- AGCTCTAGATGGTCACTGTC-3′. The following human VCAM-1-specific primers were synthesized: sense primer, 5′- CATTTGACAGGCTGGAGATA-3′ and antisense primer, 5′- GAACAGGTCATGGTCACAGA-3′. Mouse GAPDH PCR primers were 5′- TCCCTCAAGATTGTCAGCAA-3′ (sense) and 5′-AGATCCACAACGGATACATT-3′ (antisense). The absence of contaminants was routinely checked by the RT-PCR assay of negative control samples without a primer addition. Samples were stored at 20 °C, after amplification.

2.6. Real-time RT-PCR

This procedure is based on the time point during cycling when amplication of the PCR product is first detected. The total RNA was extracted using a single-step guanidinium thiocyanate-phenol-chloroform method. The yield and purity of the RNA were confirmed by measuring the ratio of the absorbances at 260 and 280 nm. RNA (3 μg) was reverse transcribed using a First-Strand cDNA Synthesis kit (Thermo scientific, UT). Real-time PCR was performed with SYBR green reagent (Applied Biosystems, CA) using 30 ng template in a 20 μl reaction mixture. Primer sequences were 5′-TTCCTCCACCACCATGCAG-3′ and 5′-CCAGCCGGCAACTGTGA for MCP-1, and 5′-TGCATCCTGCACCACCAA-3′ and 5′-TCCACGATGC-CAAAGTTGTC-3′ for GAPDH.

2.7. Transfection and reporter assays

Cells (5×10^5 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 μg pGL3-NF-κB and 0.2 μg pCMV-β-gal was mixed with the Lipofectamine Plus reagent and added to the cells. After 4 h, the cells were pre-treated with stereocalpin A for 2 h followed by the addition of TNF-α for 4 h, and then lysed with 200 μl 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 μl 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 β-galactosidase activity. Each transfection was carried out in triplicate and experiments were repeated three times.

2.8. ROS production assay

ROS production was determined as described elsewhere [16]. CMH_2DCFDA (5,6-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate; acetyl ester, Molecular Probes, Eugene, OR), a redox-sensitive fluorescent dye, was used to evaluate the intracellular ROS level by flow cytometrry. VSMCs (3×10^6 cells/ml) were pre-treated with a various concentrations of stereocalpin A for 2 h, followed by addition of TNF-α (10 ng/ml) for 4 h. The cells were then stained for 15 min at 37 °C with 5 μM CMH2-DCFDA. The cells were kept on ice in the dark and at least 10,000 cells for each sample were analyzed using a Becton Dickinson FACSCalibur (BD Biosciences, San Jose, CA). The changes in the level of intracellular ROS are expressed as a percentage of TNF-α-stimulated cells not treated with stereocalpin A.

2.9. Western blot analysis

Western blot analysis was performed by a modification of the technique described previously [17]. The cells were pretreated with stereocalpin A (0.1–10 μg/ml) for 2 h. The cells were washed with medium to remove stereocalpin A and incubated with fresh growth medium containing TNF-α (10 ng/ml) for 30 min or 8 h. After treatment, the cells were washed twice in PBS and suspended in 70 μl of Buffer A [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF and Protease Inhibitor Cocktail (Sigma)] and incubated on ice. After 15 min, 0.5% Nonidet P (NP)-40 was added to lyse the cells, which were vortexed for 10 s. Then, cytosolic cell extracts were obtained after centrifuging at 1500×g for 10 min at 4 °C. The collected nuclei were resuspended in 50 μl of Buffer C [20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 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×g at 4 °C. Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Lab, Hercules, CA) with BSA as the standard. The cytosol lysates (20 μg) and nuclear extracts (40 μg) were resolved on a 7.5% SDS-polyacrylamide gel, respectively. The fractionated proteins were electrophoretically transferred to an immobilon polyvinylidene difuride membrane (Amersham, Arlington Hights, IL) and probed with the appropriate antibodies. The blots were developed using an enhanced chemoluminescence (ECL) kit (Amersham). In all immunoblotting experiments, the blots were

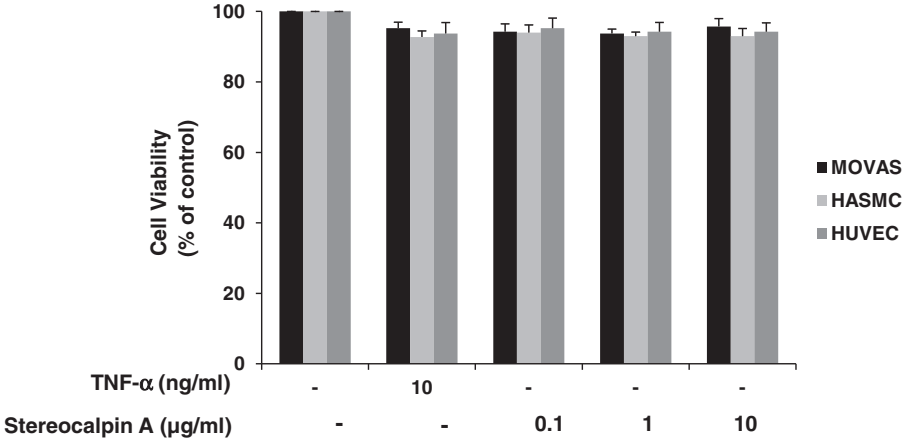

Fig. 2. Effect of stereocalpin A or TNF-α on cell viability. Cells were treated for 24 h with the indicated concentrations of stereocalpin A or TNF-α. No significant cytotoxicity of stereocalpin A or TNF-α was observed under the experimental conditions. The results are the mean ± S.E.M. of quintuplicates from a representative experiments.

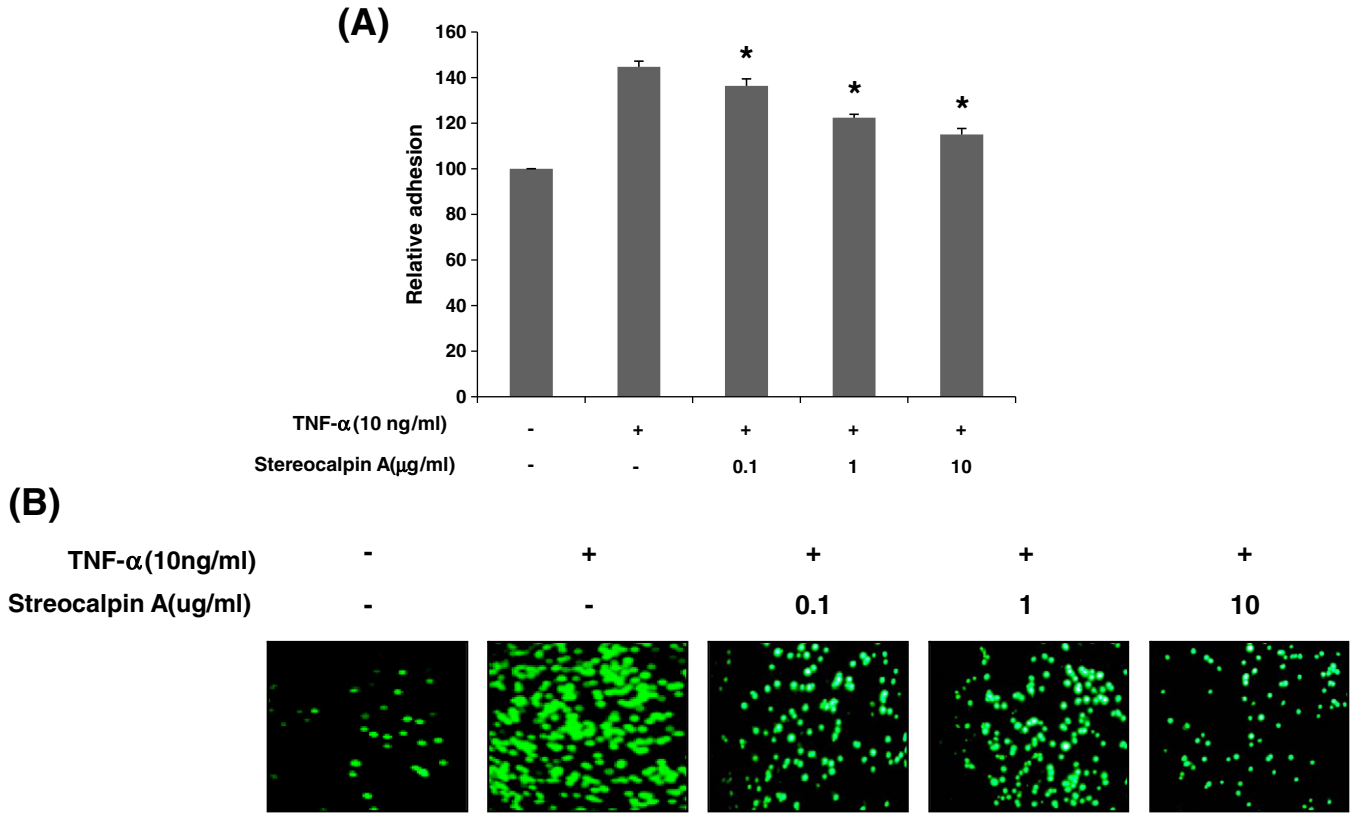

Fig. 3. Stereocalpin A inhibits adhesion of THP-1 cells to TNF-α-stimulated VSMCs.MOVAS-1 cells were pre-treated for 2 h with the indicated concentrations of stereocalpin A. The cells were washed twice with medium and incubated with TNF-α (10 ng/ml) for 6 h. The BCECF-labeled THP-1 cells were added to MOVAS-1 cells monolayer and allowed to adhere for 1 h. (A) The adhesion was measured as described in the "Materials and methods". (B) Adherent THP-1 to MOVAS-1 cells was observed under a fluorescent microscope at 100× magnification. The results are expressed as the mean ± S.E.M. of three independent experiments performed in triplicates. * Significantly different from TNF-α-stimulated cells not treated with stereocalpin A ($P<0.05$).

reprobed with an anti-β-actin antibody as a control for the protein loading.

2.10. Measurement of cell viability

VSMCs were seeded at a concentration of 2×104 cells/well in 96-well tissue culture plates and treated with various concentrations of stereocalpin or 10 ng/ml of TNF-a for the indicated times. Cell viability was measured by quantitative colorimetric assay with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], showing the mitochondrial activity of living cells. The extent of the reduction of MTT to formazan within cells was quantified by measuring the optical density at 550 nm using a Molecular Device microplate reader (Menlo Park, CA). The cell viability was expressed as the percentage of the untreated control.

2.11. Statistical analysis

Each result is reported as means ± S.E.M. One-way analysis of variance was used to determine significance among groups, after which the modified *t* test with the Bonferroni correction was used for comparison between individual groups. The significant values are represented by an asterisk. (*$P<0.05$).

3. Results

3.1. Effect of sterocalpin A on cell viability

To examine the effect of stereocalpin A on cell viability, MOVAS-1 cells, HASMCs and HUVECs were treated for 8 h with the indicated concentrations of stereocalpin A or TNF-α. As shown in Fig. 2, no significant cytotoxicity of stereocalpin A or TNF-α was observed. In addition, the concentration of stereocalpin A or TNF-a used in our experiments did not alter cell viability as assessed by trypan blue staining and morphology of the muscle cells, but concentrations > 10 μg/ml were found to be cytotoxic (data not shown).

3.2. Effect of stereocalpin A on vascular cell adhesion molecule expression and adhesion of monocyte to muscle cells

Our initial experiments were designed to examine the adherence of leukocytes to cytokine-activated vascular smooth muscle cells (VSMCs) pretreated with stereocalpin A. Cytokine treatment results in the adhesive capacity of vascular smooth muscle cells, inducting adhesion molecule expression. As shown in Fig. 3, treatment of confluent MOVAS-1 cells with 10 ng/ml TNF-α for 6 h caused an almost 1.5-fold increase in adhesion of THP-1 monocytic cells compared with adhesion of THP-1 cells to un-stimulated MOVAS-1 cells. The binding of THP-1 cells to MOVAS-1 cells was decreased in a dose-dependent manner by pre-incubation of the cells for 2 h with increasing concentrations of stereocalpin A.

In order to investigate the mechanism whereby stereocalpin A decreased the binding of monocyte to VSMCs, we examined the expression of adhesion molecules, which are known to support the binding of monocytes. MOVAS-1 cells were pre-incubated without or with various concentrations of stereocalpin A for 2 h and then stimulated with TNF-α (10 ng/ml). Preincubation with stereocalpin A did not affect the basal level of adhesion molecule expression. As detected by ELISA, the cell surface expression of ICAM-1 and VCAM-1 was induced by TNF-α (Fig. 4A). However, pretreatment with stereocalpin

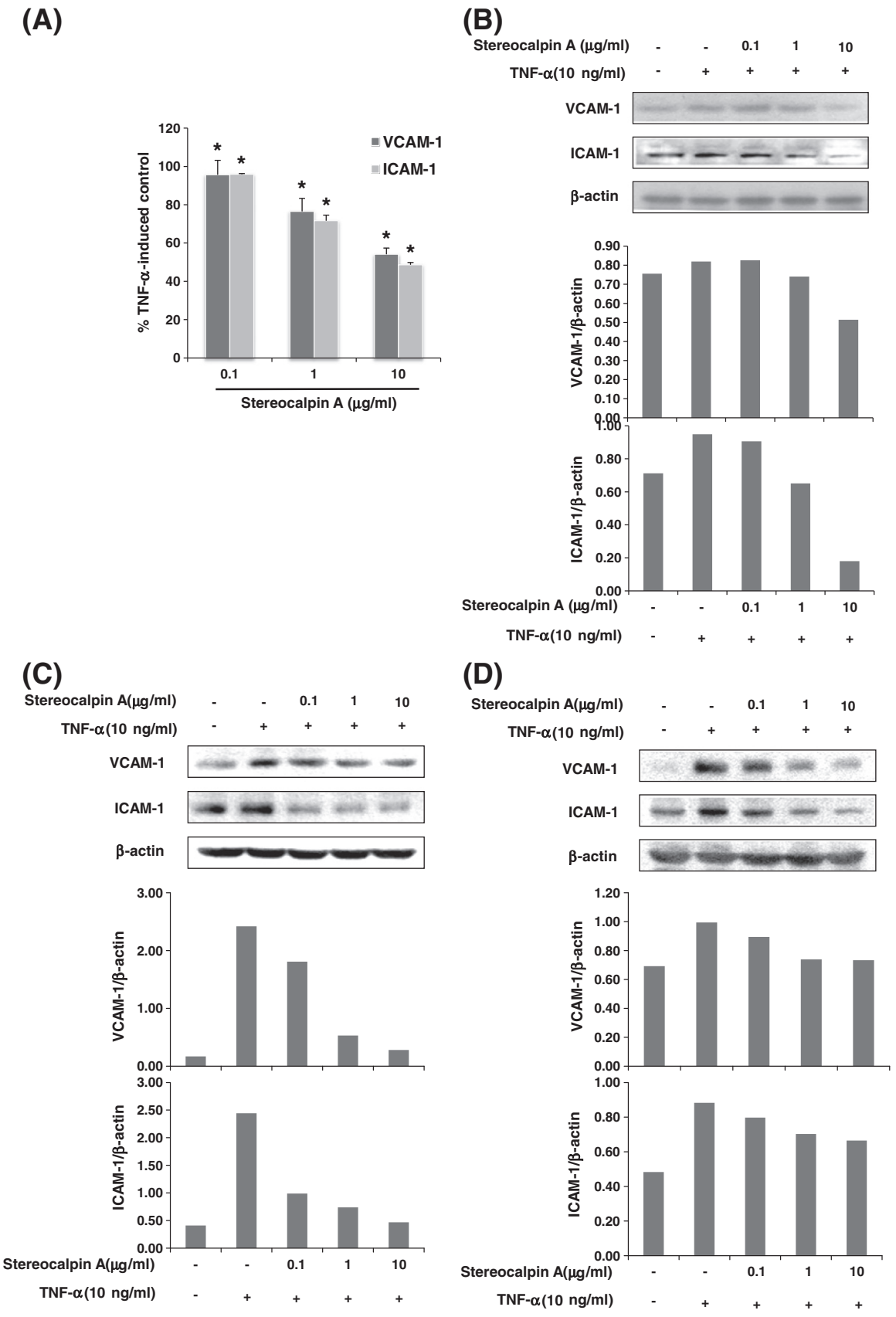

Fig. 4. Effect of stereocalpin A on TNF-α-stimulated adhesion molecule expression.(A) Expression of VCAM-1 and ICAM-1 in MOVAS-1 cells after pre-incubation with the indicated concentrations of stereocalpin A was measured by ELISA. The data are expressed as a percentage of TNF-α-induced adhesion molecule expression. Data are expressed as the mean ± S.E.M of 3 experiments. * Significantly different from TNF-α-stimulated cells not treated with stereocalpin A ($P<0.05$). The VCAM-1 and ICAM-1 protein levels in MOVAS-1 cells (B), HASMCs (C) and HUVECs (D) were determined by Western blot assay. The β-actin protein level was considered as an internal control. The results illustrated are from a single experiment, and are representative of three separate experiments. The intensity of the bands was quantitated by densitometry. The levels of VCAM-1 and ICAM-1 expression are in arbitrary units, and data are normalized to respective amount of β-actin protein.

A resulted in a significant reduction in the TNF-α-induced ICAM-1 and VCAM-1 expression in a concentration-dependent manner. In addition, stereocalpin A remarkably decreased TNF-α induced ICAM-1 and VCAM-1 protein level (Fig. 4B). Significant reduction of the expression of the two adhesion molecules was observed at 10 μg/ml. Our data also show that sterocalpin A similarly suppressed TNF-α-induced ICAM-1 and VCAM-1 expression in a concentration-dependent fashion in primary human aortic smooth muscle cells (HASMCs) and human vascular endothelial cells (HUVECs) (Fig. 4C and D). The inhibitory effect of stereocalpin A was selective, because stereocalpin A did not affect the expression of β-actin. Taken together, these results suggest that stereocalpin A is effective in blocking the induced level of expression of ICAM-1 and VCAM-1.

3.3. Effect of stereocalpin A on ICAM-1 and VCAM-1 mRNA induction by TNF-α

Next, we were interested in determining whether the interference of stereocalpin A with TNF-α-induced adhesion molecule expression also occurs at the transcription level. To examine gene transcription level of mRNA, total cellular RNA was isolated from VSMCs and analyzed by RT-PCR using ICAM-1 and VCAM-1-specific probes. VSMCs were pre-treated with various concentrations of stereocalpin A for 2 h, then stimulated with TNF-α for 4 h. As shown in Fig. 5A, treatment of VSMCs with stereocalpin A significantly inhibited the induction of ICAM-1 and VCAM-1 mRNAs. These results suggest that the ICAM-1 and VCAM-1 expression induced by TNF-α is post-transcriptionally modulated by stereocalpin A.

Stereocalpin A similarly inhibited the expression of ICAM-1 and VCAM-1 mRNA in a dose-dependent fashion in HASMCs and HUVECs (Fig. 5B and C), indicating that such phenomena are not restricted to MOVAS-1 cells and are more likely to be features of vascular cells in general. The most work concentrates on using MOVAS-1 cells as a vascular cell model.

3.4. Inhibition of TNF-α-induced NF-κB activation by stereocalpin A

It is well documented that NF-κB is ubiquitous transcription factor that plays a crucial role in adhesion molecule expression [15,18-20]. We therefore examined the effect of stereocalpin A on NF-κB transcriptional activation. MOVAS-1 cells were treated with various concentrations of stereocalpin A for 2 h before stimulation with TNF-α for 4 h. We used transcriptional activation assays to determine whether stereocalpin A affects NF-κB dependent transcription. Stimulation by TNF-α resulted in an approximately 3.6-fold increase in luciferase activity, and this increase was significantly decreased by stereocalpin A (Fig. 6A). Since the p65 subunit of NF-κB has been demonstrated to exert critical activity in the transcription of many inflammatory genes, we also investigated the effect of stereocalpin A on the expression of p65 NF-κB protein. As shown in Fig. 6B, pretreatment of VSMCs with stereocalpin A reduced p65 NF-κB translocation to the nuclear fraction, suggesting that stereocalpin A inhibits the TNF-α-induced nuclear translocation of NF-κB. These data suggest that stereocalpin A inhibits the TNF-α-induced nuclear translocation of NF-κB.

The translocation of NF-κB to the nucleus is preceded by the phosphorylation, ubiquitination, and proteolytic degradation of IκBα [21,22]. To determine whether stereocalpin A affects TNF-α-induced degradation of IκBα, we performed Western blot assay to detect the expression of IκBα protein in MOVAS-1 cells (Fig. 6B). Analysis of cell extracts using IκBα-specific antibody showed that stimulation with TNF-α treatment caused rapid degradation of IκBα as compared to untreated, but in cells pre-incubated by stereocalpin A TNF-α failed to degradation of IkBα. These results further demonstrate that stereocalpin A inhibits TNF-α-induced NF-κB activation. Similar results were also found in both HASMCs and HUVECs (Fig. 6C and D).

Taken together, our data indicate that stereocalpin A inhibits the activation of NF-κB, which might be associated with the blocking of TNF-α-induced adhesion molecule production by stereocalpin A.

3.5. Effect of stereocalpin A on TNF-α-induced phosphorylation of MAPs and Akt in smooth muscle cells

It has been reported that the activation of the MAP kinase (MAPK) in response to TNF-α treatment increases the expression of adhesion molecules [23,24]. The present data also showed that the stereocalpin A treatment influenced vascular CAM expression. Therefore, the p38 MAPK, ERK1/2 and JNK kinase pathways were examined to determine if stereocalpin A interferes with the expression of adhesion molecules by TNF-α. As shown in Fig. 7A, the levels of activation of p38 MAPK, ERK1/2 and JNK in untreated cells were clearly increased by TNF-α treatment. However, the induced MAPK activity was significantly reduced by pre-treatment with stereocalpin A for 2 h.

A previous study has shown that Akt has been implicated in the regulation of adhesion molecule expression and NF-κB activation induced by TNF-α [25]. Therefore, we further investigated the effect of stereocalpin A on TNF-α-induced Akt activation. As shown in Fig. 7A, stereocalpin A treatment resulted in the inhibition of TNF-α-induced Akt activation in a dose dependent manner. Stereocalpin A similarly inhibited the phosphorylation of MAPK and Akt in HASMCs (Fig. 7B). These results suggest that stereocalpin A may reduce TNF-α-induced ICAM-1 and VCAM-1 expression by decreasing the activation of MAPK and Akt pathways.

3.6. Effects of stereocalpin A on the ROS production and MCP-1 expression in TNF-α-stimulated smooth muscle cells

Because it has been suggested that TNF-α induced ROS production activates NF-κB in vascular cells [26,27] and the present data showed that treatment of cells with stereocalpin A significantly attenuated the activation of NF-κB, the effect of stereocalpin A on the production of TNF-α-induced ROS was investigated. MOVAS-1 cells were pretreated with stereocalpin A for 2 h and then stimulated with TNF-α. Stereocalpin A significantly reduced the production of TNF-α-induced ROS in a concentration-dependent manner (Fig. 8A). At the highest concentration of stereocalpin A tested (10 μg/ml), the ROS production was reduced to approximately 60%. These results suggest that stereocalpin A has an antioxidant activity.

The objectives of the next series of experiments were to investigate the effect of stereocalpin A on the MCP-1 expression in VSMCs, because MCP-1 displays chemotactic activity for monocytes and has been implicated in the pathogenesis of diseases characterized by monocytic infiltrates, like atherosclerosis [28], and our data shows that stereocalpin A treatment resulted in a significant decrease in monocyte adhesion (Fig. 8B).

4. Discussion

In the present study, our data demonstrate that stereocalpin A obtained from *Stereocaulon alpium*, a polar lichen, has an antiinflammatory activity which is not reported for this compound so far.

The presence of cytokine such as TNF-α in the atherosclerotic lesion could contribute to the expression of adhesion molecules. Vascular smooth muscle cells express the cellular adhesion molecules VCAM-1 and ICAM-1 in many inflammatory diseases including atherosclerosis. The expression of such adhesion molecules in VSMCs was described as prominent in the fibrous caps of advanced atherosclerotic plaques and was also associated with disease severity [29]. Thus, pharmacological agents that block the expression of these adhesion molecules have the potential to inhibit inflammatory diseases, such as atherosclerosis. To our knowledge, this is the first report

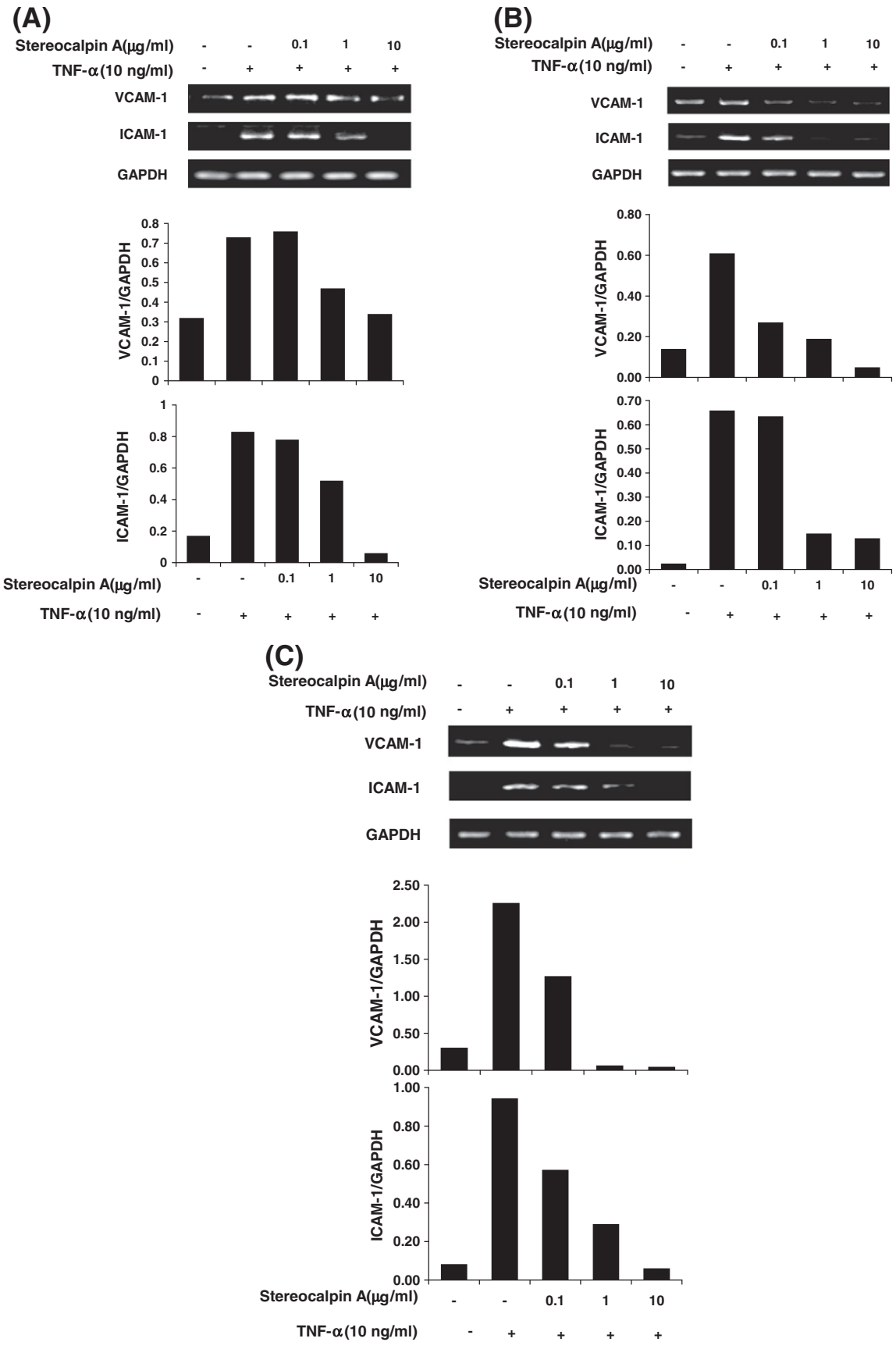

Fig. 5. Stereocalpin A inhibits TNF-α-induced up-regulation of VCAM-1 and ICAM-1 mRNA levels in VSMCs. MOVAS-1 cells (A), HASMCs (B) and HUVECs (C) were pre-incubated with indicated concentrations of stereocalpin A for 2 h and stimulated with TNF-α (10 ng/ml) for 4 h. Levels of the mRNA for adhesion molecules were determined by RT-PCR. GAPDH served as a housekeeping gene. The results illustrated are from a single experiment, and are representative of three separate experiments. Expression of VCAM-1 and ICAM-1 mRNA are in arbitrary units, and data are normalized to respective amount of GAPDH mRNA. The results illustrated are from a single experiment, and are representative of three separate experiments.

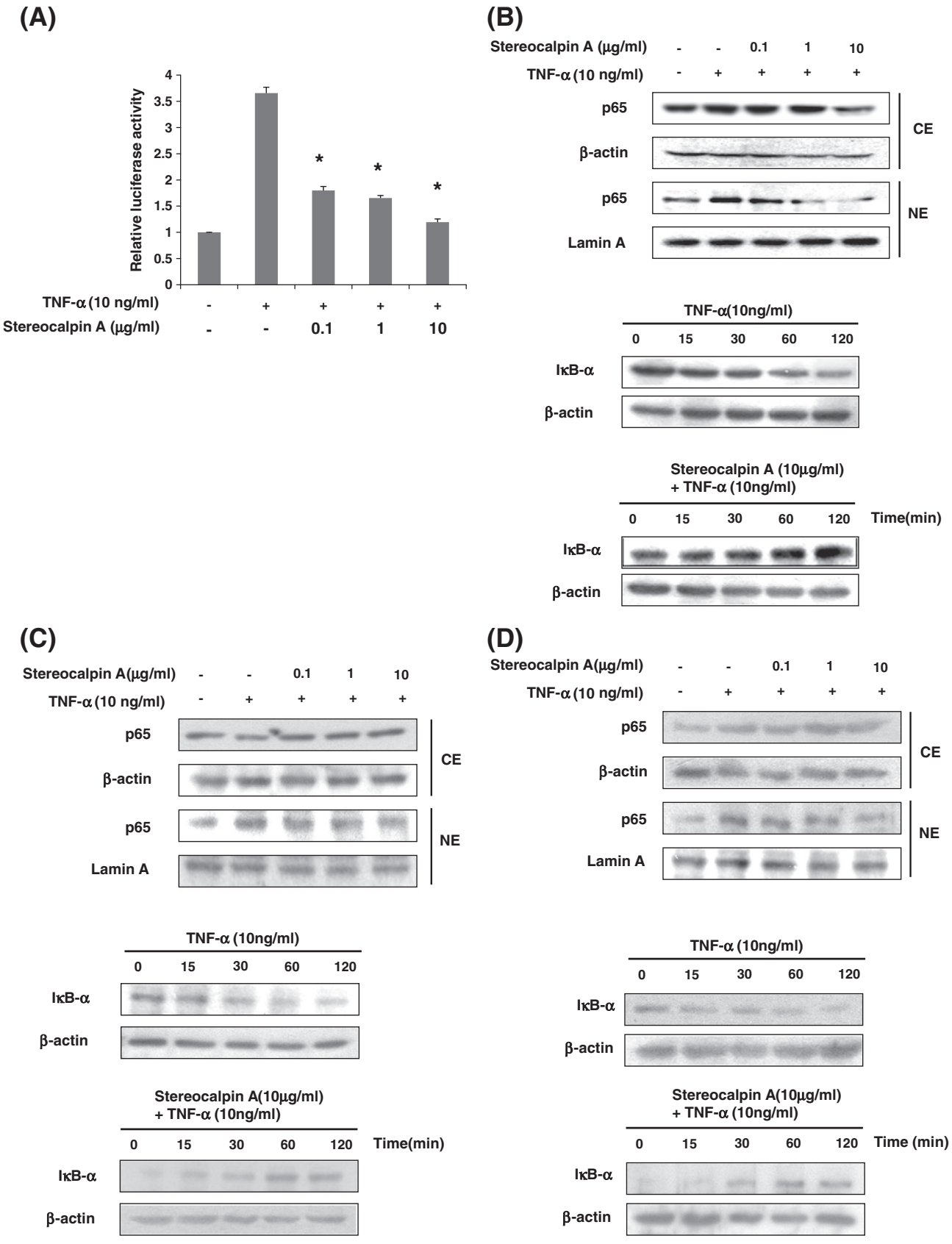

Fig. 6. Effects of stereocalpin A on NF-κB activation and IκBα degradation in TNF-α-stimulated VSMCs. NE, nuclear extracts; CE, cytoplasmic extracts. (A) MOVAS-1 cells were transfected with a pGL3-NFκB-Luc reporter plasmid and pCMV-β-gal, pre-treated with various concentrations of stereocalpin A for 2 h, and stimulated with TNF-α (10 ng/ml) for 4 h. The results are mean ± S.E.M of 3 experiments. $^{*}P<0.05$, significantly different from the group treated with TNF-α. MOVAS-1 cells (B), HASMCs (C) and HUVECs (D) were pre-incubated with or without various concentrations of stereocalpin A for 2 h, then treated with TNF-α (10 ng/ml) for 4 h. Cytoplasmic and nuclear levels of NF-κB p65 were detected by Western blotting to analyze the translocation of NF-κB. IκBα degradation was also analyzed by Western blot with anti-IκBα antibody. β-actin and lamin A were used as loading controls for whole cell lysates and nuclear protein fractions, respectively. The results illustrated are from a single experiment, and are representative of three separate experiments.

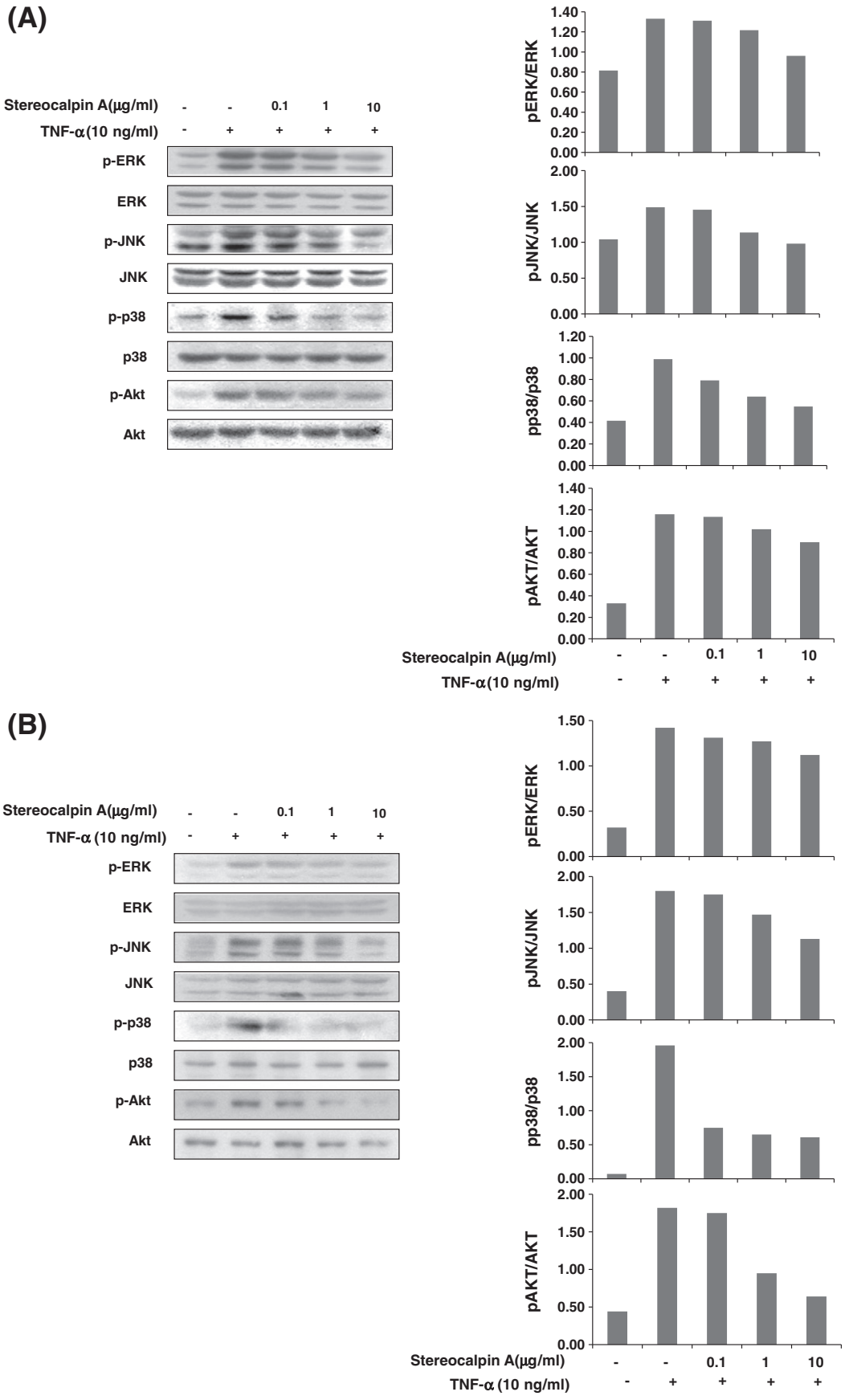

Fig. 7. Effect of stereocalpin A on pp38, p-JNK, p-ERK and Akt activation in TNF-α-stimulated VSMCs. MOVAS-1 cells (A) and HASMCs (B) were pre-treated with the indicated concentration of stereocalpin A for 2 h and then incubated with TNF-α (10 ng/ml) for 30 min. The whole cell lysates were analyzed by Western blot. A typical result from three independent experiments is shown. Immunoblot band intensities were measured by scanning densitometry. The relative intensities were expressed as the ratio of phospho-MAPK or phospho-Akt to total MAPK or Akt.

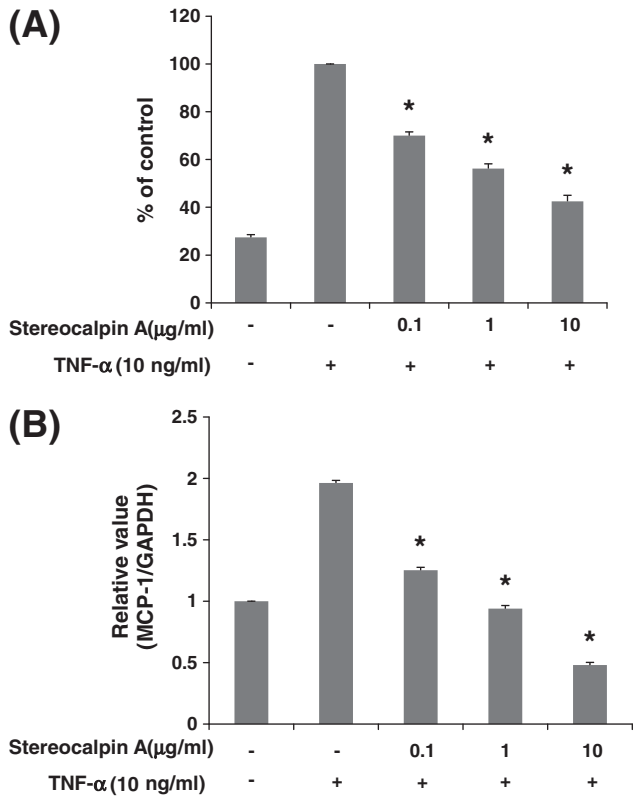

Fig. 8. Effects of stereocalpin A on the production of ROS and MCP-1 expression in TNF-α-stimulated VSMCs. MOVAS-1 cells were treated with stereocalpin A at the concentrations indicated for 2 h followed by the stimulation with TNF-α (10 ng/ml) for 4 h. (A) The level of ROS was measured as described in Materials and methods. The results are mean ± S.E.M of quintuplicates from a representative experiment. (B) The expression of MCP-1 mRNA levels was measured by real-time RT-PCR. Data are expressed as the mean ± S.E.M of 3 experiments. *$P<0.05$, significantly different from the group treated with TNF-α.

showing the inhibitory effect of stereocalpin A on adhesion molecule expression in vascular smooth muscle cells.

In this study, we evaluated the effects of stereocalpin A on expression of the muscle adhesion molecule VCAM-1 and ICAM-1 as well as on monocyte adhesion to VSMCs. Stereocalpin A prevented induction of expression of adhesion molecules in a concentration-dependent manner after stimulation with the inflammatory cytokine, TNF-α, at the level of protein and mRNA. In corroboration of these results, adhesion of monocytes to the muscle monolayer was significantly inhibited by stereocalpin A. It has been known that the expression of adhesion molecules on VSMCs may facilitate the accumulation of transmigrated leukocytes within vascular wall [6]. Interactions of VSMCs with monocytes via cell adhesion molecules may contribute to the inflammatory reaction in the vascular wall and be involved in the progression of atherosclerotic plaques [5]. Based on these findings, our data suggest that the changes in adhesion molecules by stereocalpin A provide the vascular muscle cell with a mechanism of regulating cell–cell interactions during recruitment of monocytes.

MAP kinases are a family of serine-threonine kinases, participating in the regulation of cell adhesion molecules expressed on cells in response to external stimuli including TNF-α [23,24]. There is much evidence that cells may utilize different MAPK signaling pathways for TNF-α-induced expression of adhesion molecules and that the MAPK signaling pathways are differently involved in response to anti-inflammatory compounds. Furthermore, Akt has been known to be involved in the NF-κB activation and adhesion molecule expression induced by TNF-α [25]. Therefore, we have examined the effect of stereocalpin A on Akt pathway and JNK, p38 MAPK and ERK1/2 pathways which are three well-characterized subtypes of MAPKs. Our data demonstrate that stereocalpin A significantly inhibited phosphorylation of JNK, p38 MAPK, ERK1/2 and Akt phosphorylation in TNF-α-stimulated VSMCs. Thus, these results suggest that stereocalpin A treatment inhibits TNF-α-induced adhesion molecule expression through inhibition of MAPK and Akt activation. As the present data demonstrated that stereocalpin A inhibited TNF-α-induced transcript levels for the adhesion molecules, it may be interfering at an early stage of signaling event induced by TNF-α. This suggests that stereocalpin A may inhibit the expression of the cell adhesion molecules by interfering with the transcription of their respective genes. It may inhibit either the initiation of transcription or the stability of the mRNAs encoding these molecules. It has been also shown that activation of transcription factor NF-κB by TNF-α is required for the transcriptional activation of muscle cell adhesion molecules [30]. Thus, NF-κB is believed to play an important role in the regulation of inflammatory response. NF-κB activation was associated with the phosphorylation and degradation of IκB-α and the nuclear translocation of p65 [21,22]. In addition, it has been reported that NF-κB is essential to the gene expression of cell adhesion molecules [31]. Therefore, the decreased expression of adhesion molecules prompted us to examine the effect of stereocalpin A on NF-κB, a key transcription factor implicated in the regulation of a variety of genes participating in immune and inflammatory responses, including gene encoding VCAM-1 and ICAM-1. The present data show that stereocalpin A inhibited TNF-α-induced NF-κB activation through inhibition of IκB kinase activation and subsequent IκBα degradation. However, our results do not eliminate the possible involvement of alternative transcription factors and signaling pathways, because AP-1, SP-1, GATA-2, and IRF-1 are known to be involved in gene expression of adhesion molecules [32–35]. Therefore, the present data suggest that inhibitory effect of stereocalpin A on adhesion molecule expression is at least partially mediated through suppression of the NF-κB transcription factor.

It has been shown that many of the effects of cytokines on vascular cells involve increases in reactive oxygen species (ROS) [36]. Inflammatory cytokine like TNF-α may increase ROS that is generated at sites of inflammation and injury. In addition, there is growing evidence for the involvement of ROS in atherosclerotic plaque development [37]. In the present study, pretreatment with stereocalpin A significantly inhibited the TNF-α induced ROS production in vascular muscle cells, indicating that stereocalpin A has an antioxidant activity. It has been demonstrated that ROS activates various transcription factors in cultured vascular cells and may function as a signaling molecule in various pathways leading to MAPK, Akt, NF-κB activation [38–43]. Furthermore, adhesion molecule expression has been implicated to occur by stimulation of NF-κB signaling pathway through the induction of ROS [44]. These observations strongly indicate a possible connection between ROS and this transcription factor. Indeed, our current data show that blockage of NF-κB activity by stereocalpin A would impair the TNF-α-induced CAM expression. Therefore, it is plausible that the inhibitory effect of stereocalpin A on the TNF-α induced expression of adhesion molecules and activation of NF-κB, Akt and MAPK is due to its antioxidative properties.

As the results described previously, we observed a significant decrease in monocyte adhesion at concentrations of sterocalpin A that inhibited ICAM-1 and VCAM-I expression on VSMCs. This reduction in the expression of adhesion molecules may be responsible for the observed effect on monocyte adhesion; however, it is also possible that stereocalpin A inhibits expression of other NFκB-dependent genes in smooth muscle cells such as MCP-1 that may be atherogenic, in part by promoting the firm adhesion of macrophage to smooth muscle cell that facilitates chemotactic transmigration [45]. Thus, VSMC expression of MCP-1 may be a significant contributor to the extensive accumulation of macrophages within the vessel wall. We examined the levels of MCP-1 mRNA in smooth muscle cells following a 4 h TNF-α treatment and found detectable decrease in MCP-1 mRNA levels upon treatment with sterocalpin A.

In summary, the results of the present study demonstrate that stereocalpin A prevents the expression of adhesion molecules and MCP-1 and reduces monocyte adhesion to TNF-α-stimulated VSMCs. These results suggest a pharmacological activity of stereocalpin A in vascular muscle cells. In addition, the effect of stereocalpin A is resulting from the suppression of ROS production, MAPK pathways, NF-κB and Akt activation. Thus, stereocalpin A is proposed as an effective new anti-inflammatory agent that may prevent the advancement of atherosclerotic lesions.

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