Life Sciences 90 (2012) 396–406

Contents lists available at SciVerse ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Ohioensin F suppresses TNF-α-induced adhesion molecule expression by inactivation of the MAPK, Akt and NF-κB pathways in vascular smooth muscle cells

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ARTICLE INFO

Article history:
Received 3 August 2011
Accepted 19 December 2011

Keywords:
Ohioensin F
Anti-inflammation
Vascular cell adhesion molecule
MAPK
NF-κB
Akt

ABSTRACT

Aims: The expression of cell adhesion molecules on vascular smooth muscle cells is central to leukocyte recruitment and progression of atherosclerotic disease. Ohioensin F, a chemical compound of the Antarctic moss *Polyerichastrum alpinum*, exhibited inhibitory activity against protein tyrosine phosphatase 1B and antioxidant activity. However, published scientific information regarding other biological activities and pharmacological function of ohioensin F is scarce. In the present study, we aimed to examine the in vitro effects of ohioensin F on the ability to suppress TNF-α-induced adhesion molecule expression in vascular smooth muscle cells (VSMCs).

Main methods: The inhibitory effect of ohioensin F on TNF-α-induced upregulation in expression of adhesion molecules was investigated by enzyme-linked immunosorbent assay, cell adhesion assay, RT-PCR, western blot analysis, immunofluorescence, and transfection and reporter assay, respectively.

Key findings: Pretreatment of VSMCs with ohioensin F at nontoxic concentrations of 0.1–10 μg/ml dose-dependently inhibited TNF-α-induced expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1). In addition, ohioensin F suppressed adhesion of THP-1 monocytes to TNF-α-stimulated VSMCs. Ohioensin F reduced TNF-α-induced production of intracellular reactive oxygen species (ROS) and phosphorylation of p38, ERK, JNK and Akt. Finally, ohioensin F inhibited TNF-α-induced CAM mRNA expression and NK-κB translocation.

Significance: These results suggest a new mechanism of ohioensin F's anti-inflammatory action, owing to the negative regulation of TNF-α-induced adhesion molecule expression, monocyte adhesion and ROS production in vascular smooth muscle cells. Our finding also supports ohioensin F as a potential pharmacological, anti-inflammatory molecule that has a protective effect on the atherosclerotic lesion.

Introduction

Atherosclerosis is accompanied by increased levels of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) or intracellular adhesion molecule-1 (ICAM-1) in the development of atherosclerosis and plaque instability. Several lines of evidence support a crucial role of vascular smooth muscle cell (VSMCs) at various process of atherosclerosis (Lusis, 2000; Owens et al., 2004; Falk, 2006). 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 (Braun et al., 1999). The adhesion of leukocyte to VSMC during atherosclerotic progression is mainly mediated by cell adhesion molecules including VCAM-1 and ICAM-1. The expression of these molecules was detected on smooth muscle cells of plaques with an increase after vascular injury and could promote leukocyte recruitment to the atherosclerotic vascular wall (Jang et al., 1994; Libby and Li, 1993). In addition, TNF-α, a well-known pro-inflammatory cytokine, induces ICAM-1 and VCAM-1 expression on vascular cells (Huo and Ley, 2001). Therefore, blocking the expression of these adhesion molecules on VSMCs, thus inhibiting the interaction between leukocytes and vascular cells, may be beneficial in blunting detrimental inflammatory process in atherosclerosis.

There has been increasing interest in the flora and fauna of the Antarctic in recent years. In addition, recent exceptional growth in human exposure to compounds derived from plant sources has lead to a resurgence of scientific interest in their biological effects (Acuna et al., 2002). While it is generalized that biological activity of moss can depend on ecological conditions and methodology sampling species, few samples have been published. Moreover, although only a small number of moss species are found in the Antarctic region, the biological effects of these mosses are not well known.

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doi:10.1016/j.lfs.2011.12.017

Ohioensin F, a new benzonaphthoxanthenone, has been isolated from MeOH extract of Antarctic moss *Polyerichastrum alpinum* (Seo et al., 2008). Ohioensin F is suggested to be derived by condensation of *o*-hydroxycinnamate with hydroxylated phenanthrenes or 9,10-dihydrophenanthrene units (Fig. 1). Recently, ohioensin F has been reported to have inhibitory activity against protein tyrosine phosphatase 1B and antioxidant activity (Seo et al., 2008; Bhattarai et al., 2009). However, the literature survey indicates that no reports are available regarding the effect of ohioensin F on the regulation of adhesion molecule expression.

In this study, we examined the effects and mechanisms of action of ohioensin F in the context of adhesion molecule accumulation induced by TNF-α. We found that ohioensin F inhibited cell adhesion through the inhibition of ICAM-1 and VCAM-1 expression, at least in part, by inactivating MAPK, Akt and NF-κB signaling pathways and blocking ROS production.

Materials and methods

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, TNFR1, lamin A and β-actin were purchased from Abcam Inc., USA.

Cell culture

Mouse vascular smooth muscle cell line MOVAS-1 was purchased from ATCC (Rockville, MD) and were grown in DMEM medium 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. Primary human aortic smooth muscle cells (HASMC) were purchased from Clonetics Corp. (San Diego, CA). HASMC were cultured in MCDB 131 medium supplemented with 5% fetal calf serum, 10 ng/ml recombinant human epidermal growth factor, 2 ng/ml basic fibroblast growth factor, and 5 μg/ml insulin. 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 (Tsuchiya et al., 1980). 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.

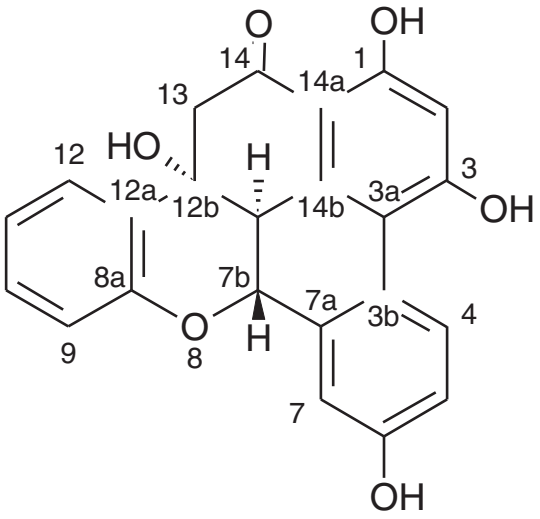

Fig. 1. Chemical structure of ohioensin F.

Preparation of peripheral blood mononuclear cells

Human monocytes were isolated from buffy coats prepared from healthy volunteer donors. Peripheral blood mononuclear cells isolated by Ficoll–Hypaque density gradient centrifugation were seeded at 6×10^6/2 ml in 12-well plates in RPMI 1640 medium and supplemented with 10% heat-inactivated human AB serum (Nabi Biopharmaceuticals, Rockville, MD) 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine. The cell cultures were incubated at 37 °C and 5% CO_2 overnight, after which nonadherent cells were removed by repeated gentle washing with warm medium. The cells were further incubated for 4 d in fresh medium before manipulation. More than 95% of the adherent cells obtained with this technique were $CD14^+$ macrophages.

Cell adhesion assay

The methodology used for the adhesion assays is described elsewhere (Chen et al., 2001). Briefly, VSMCs, which were grown in 96 well plates, were pretreated with ohioensin F (0.1–10 μg/ml) for 2 h. The cells were washed with medium to remove ohioensin F and incubated with fresh growth medium containing TNF-α (10 ng/ml) for 8 h. The medium was removed from the wells and BCECF-labeled THP-1 cells or human peripheral blood monocytes (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 the number of adherent cells were determined by measuring the fluorescence intensity using a Cytoflour 2350 (Millipore, Bedford, MA). Increase in monocytes adhesion upon stimulation of VSMCs with TNF-α was calculated in relation to the basal adhesion of THP-1 cells or human peripheral blood monocytes to non-stimulated VSMCs that was set to 100.

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 (Mo et al., 2007). MOVAS-1 cells were seeded at a concentration of 2×10^4 cells/well in 96-well gelatin-coated plates, cultured to confluence and pretreated with ohioensin F (0.1–10 μg/ml) for 2 h at 37 °C. These pretreated cells were washed with medium to remove ohioensin F and then incubated with fresh growth medium containing TNF-α (10 ng/ml) for 8 h to determine the expression 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.

Immunofluorescence

MOVAS-1 cells, which were grown on 22-mm diameter glass coverslips, were pretreated with ohioensin F (0.1–10 μg/ml) for 2 h, followed by addition of TNF-α (10 ng/ml) for 4 h. Cells were washed in PBS and fixed with 3.7% formaldehyde in PBS for 15 min at room temperature, and washed in PBS. The ice-cold methanol were added to the cells and cells were incubated at −20 °C for 10 min, and washed in PBS. They were permeabilized with 1% BSA/0.2% Triton X-100/PBS for 1 h. Thereafter, they were washed in PBS and incubated with antibody against NF-kB p65 for overnight at 4 °C. After PBS washing, cells were incubated for 1 h with anti-rabbit IgG-FITC in 1% BSA/0.05% Triton X-100/PBS. Coverslips were mounted to glass slides using ProLong Gold antifade agent containing DAPI (Invitrogen) and photographed using a fluorescent microscopy (BX51-Olympus Optical).

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 ICAM-1-specific primers were synthesized: sense primer, 5′-CCTGTTTCCTGCCTCTGAAG-3′; antisense primer, 5′-GTCTGCTGAGACCCCTCTTG-3′. The following VCAM-1-specific primers were synthesized: sense primer, 5′-CCCAAGGATCCAGA GATTCA-3′; antisense primer, 5′-TAAGGTGAGGGTGGCATTTC-3′. The GAPDH PCR primers were 5′-GGTCCTCAGTGTAGCCCAAG-3′ (sense) and 5′-AATGTGTCCGTCGTGGATCT-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.

Transfection and reporter assays

MOVAS-1 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 pretreated with ohioensin F 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.

ROS production assay

ROS production was determined as described elsewhere (Lee et al., 2007). 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 cytometry. VSMCs (3×10^6 cells/ml) were pretreated with a various concentrations of ohioensin F 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 CMH_2-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 change in the level of intracellular ROS is expressed as a percentage of TNF-α-stimulated cells not treated with ohioensin F.

Western blot analysis

Western blot analysis was performed by a modification of the technique described previously (Cho et al., 2003). The cells were pretreated with ohioensin F (0.1–10 μg/ml) for 2 h. The cells were washed with medium to remove ohioensin F 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 $1500\times g$ 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 $13{,}000\times g$ at 4 °C. Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Lab, Hercules, CA) with BSA as the standard. The whole cell 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 difluoride membrane (Amersham, Arlington Hights, IL) and probed with the appropriate antibodies. The blots were developed using an enhanced chemiluminescence (ECL) kit (Amersham). In all immunoblotting experiments, the blots were reprobed with an anti-β-actin antibody as a control for the protein loading.

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. ($^*p<0.05$).

Results

Ohioensin F inhibits TNF-α-induced vascular cell adhesion molecule expression and monocyte adhesion to muscle cells

Our initial experiments were designed to examine the adherence of leukocytes to TNF-α-activated vascular smooth muscle cells (VSMCs) pretreated with ohioensin F. TNF-α treatment results in the adhesive capacity of vascular smooth muscle cells, inducting adhesion molecule expression. As shown in Fig. 2A, treatment of confluent MOVAS-1 with 10 ng/ml TNF-α for 8 h caused an almost 1.5-fold increase in adhesion of THP-1 monocytic cells compared with adhesion of THP-1 cells to unstimulated VSMCs. The binding of THP-1 cells to VSMCs was

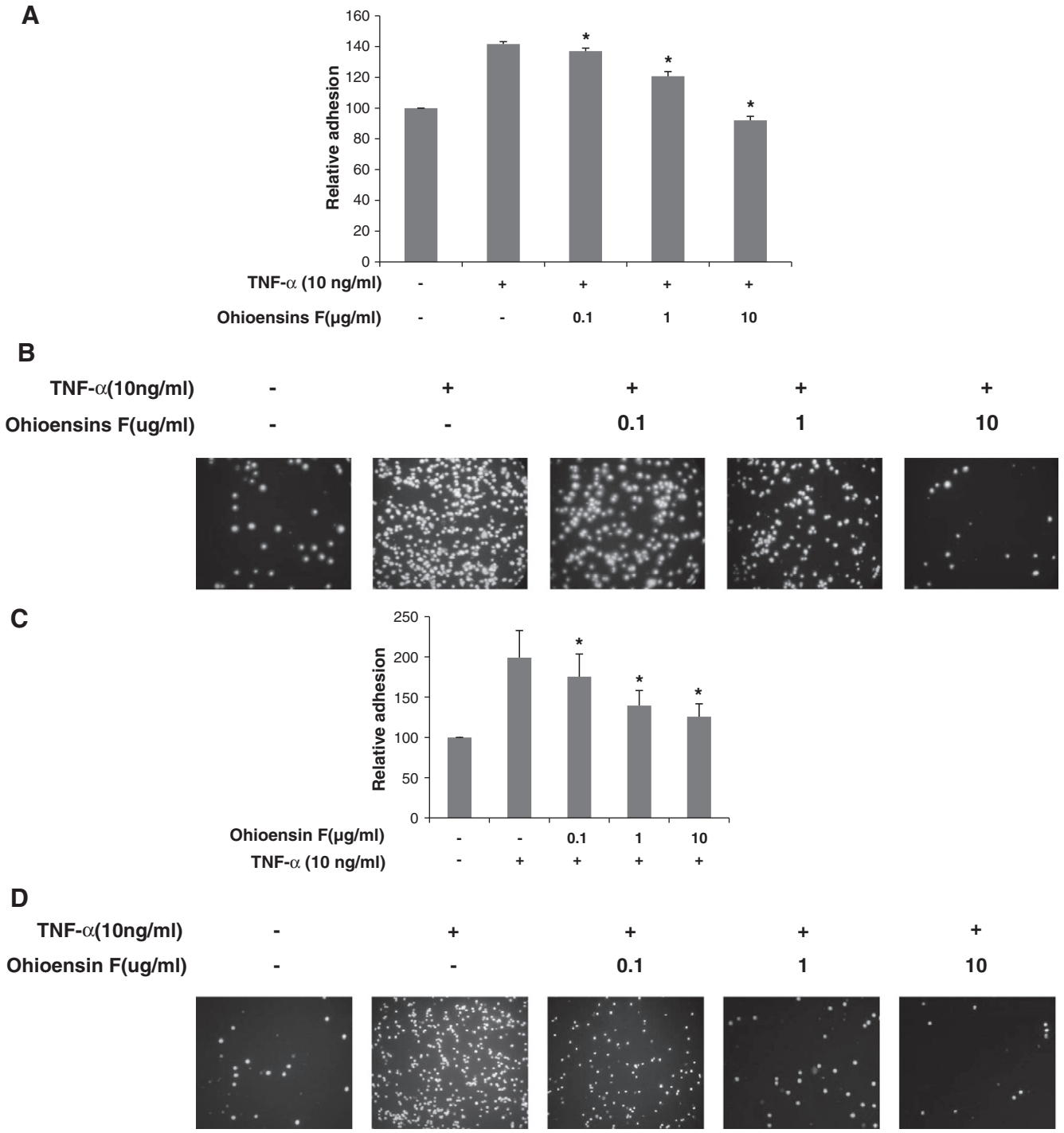

Fig. 2. Inhibition of adhesion of THP-1 cells to TNF-α-stimulated VSMCs by ohioensin F. Confluent MOVAS-1 cells (A, B) or HASMC (C, D) were pretreated for 2 h with the indicated concentrations of ohioensin F. The cells were washed twice with medium and incubated with TNF-α (10 ng/ml) for 8 h. The BCECF-labeled THP-1 cells or human peripheral blood monocytes were added to the VSMC monolayer and allowed to adhere for 1 h. The adhesion was measured as described in Materials and methods (A, C). Adherent THP-1 to VSMCs was observed under a fluorescent microscope at 100× magnification (B, D). 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 ohioensin F ($P<0.05$).

decreased in a concentration-dependent manner by preincubation of the cells for 2 h with increasing concentrations of ohioensin F (Fig. 2B). In addition, similar results were obtained when using human aortic smooth muscle cells (HASMC) and human peripheral blood mononuclear cells (Fig. 2C and D). TNF-α-induced adhesion of blood mononuclear cells to HASMC was dose-dependently reduced by 2 h of preincubation with ohioensin F prior to TNF-α treatment.

In order to investigate the mechanism whereby ohioensin F 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 and HASMC were preincubated without or with various concentrations of ohioensin F for 2 h and then stimulated with TNF-α (10 ng/ml) for 8 h. Preincubation with ohioensin F did not affect the basal level of adhesion molecule expression, and the time of incubation and concentration of ohioensin F used in these experiments had no effect on the viability as determined by trypan blue staining and morphology of the muscle cells, but concentrations >10 μg/ml were found to be cytotoxic (data not

shown). As detected by ELISA, exposure of cells to TNF-α induced strong upregulation of surface expression of ICAM-1 and VCAM-1 (Fig. 3A). However, pretreatment with ohioensin F resulted in a significant reduction in the TNF-α-induced ICAM-1 and VCAM-1 expression in a concentration-dependent manner. In addition, ohioensin F remarkably decreased TNF-α induced ICAM-1 and VCAM-1 protein level in both cell types (Fig. 3B and C). Significant reduction of the expression of the two adhesion molecules was observed at 10 μg/ml. Moreover, similar results were confirmed in HASMC. This inhibitory effect was selective, because ohioensin F did not affect the expression of β-actin. Thus, the data suggest that ohioensin F is effective in blocking the induced level of expression of ICAM-1 and VCAM-1.

Effect of ohioensin F on vascular cell adhesion molecule mRNA induction

The experiments described above demonstrated that ohioensin F significantly inhibited the VCAM-1 and ICAM-1 expression in VSMCs stimulated with TNF-α. It was possible that ohioensin F inhibited VCAM-1 and ICAM-1 expression by modulating the transcription level of adhesion molecules. To address this possibility, total cellular RNAs were isolated from VSMCs and analyzed by RT-PCR using VCAM-l or ICAM-1-specific probe. VSMCs were pretreated with various concentrations of ohioensin F for 2 h, then stimulated with TNF-α for 4 h. As shown in Fig. 4, treatment with ohioensin F significantly inhibited the induction of VCAM-1 and ICAM-1 mRNAs in MOVAS-1

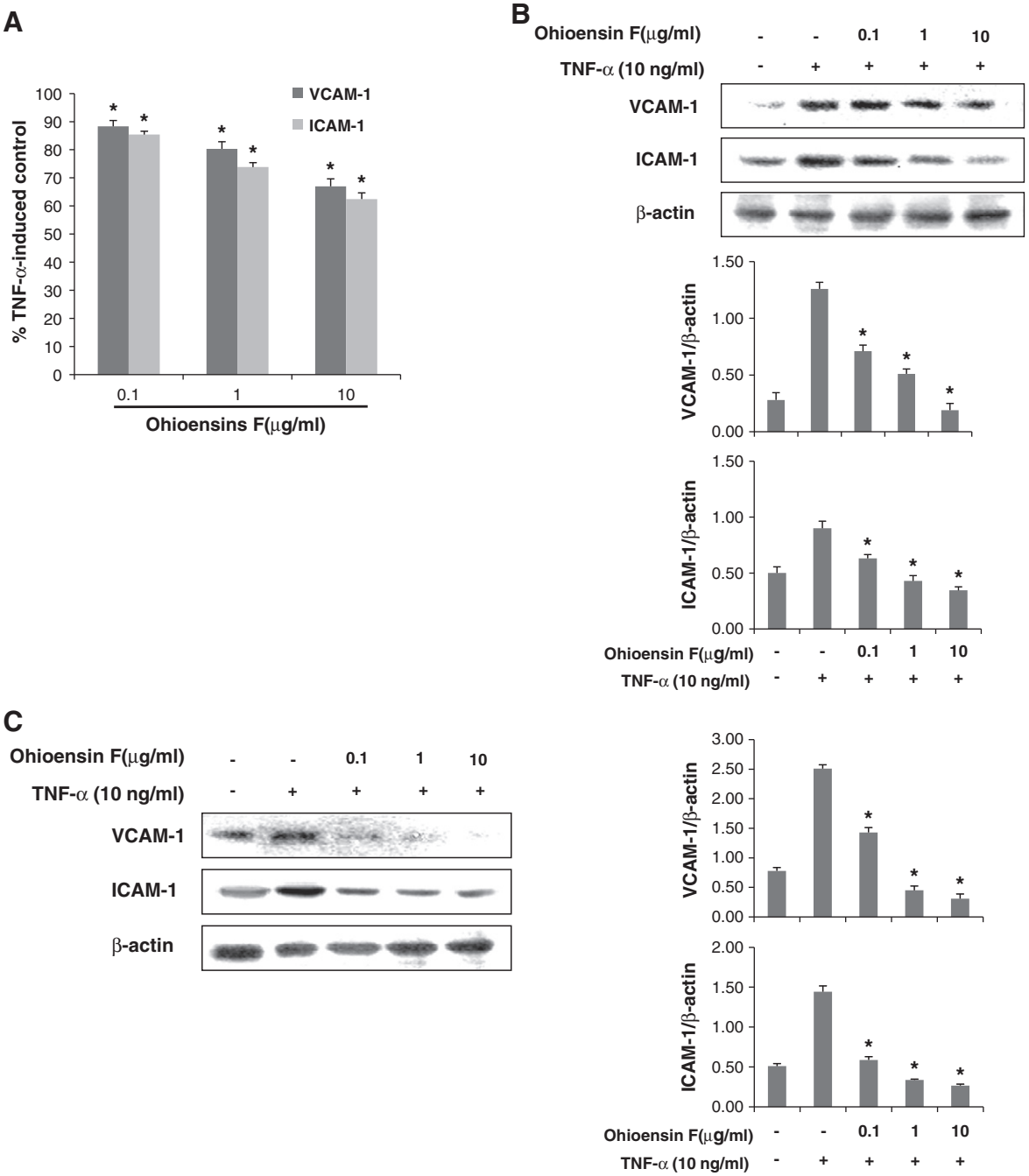

Fig. 3. Effect of ohioensin F on TNF-α-stimulated adhesion molecule expression. Expression of VCAM-1 and ICAM-1 in MOVAS-1 cells after pre-incubation with the indicated concentrations of ohioensin F was measured by ELISA (A). The data are expressed as a percentage of TNF-α-induced adhesion molecule expression. The VCAM-1 and ICAM-1 protein levels were determined in MOVAS-1 cells (B) or HASMC (C) by Western blot assay. The β-actin protein level was considered as an internal control. 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. Data are expressed as the mean ± S.E.M of 3 experiments. * Significantly different from TNF-α-stimulated cells not treated with ohioensin F ($P<0.05$).

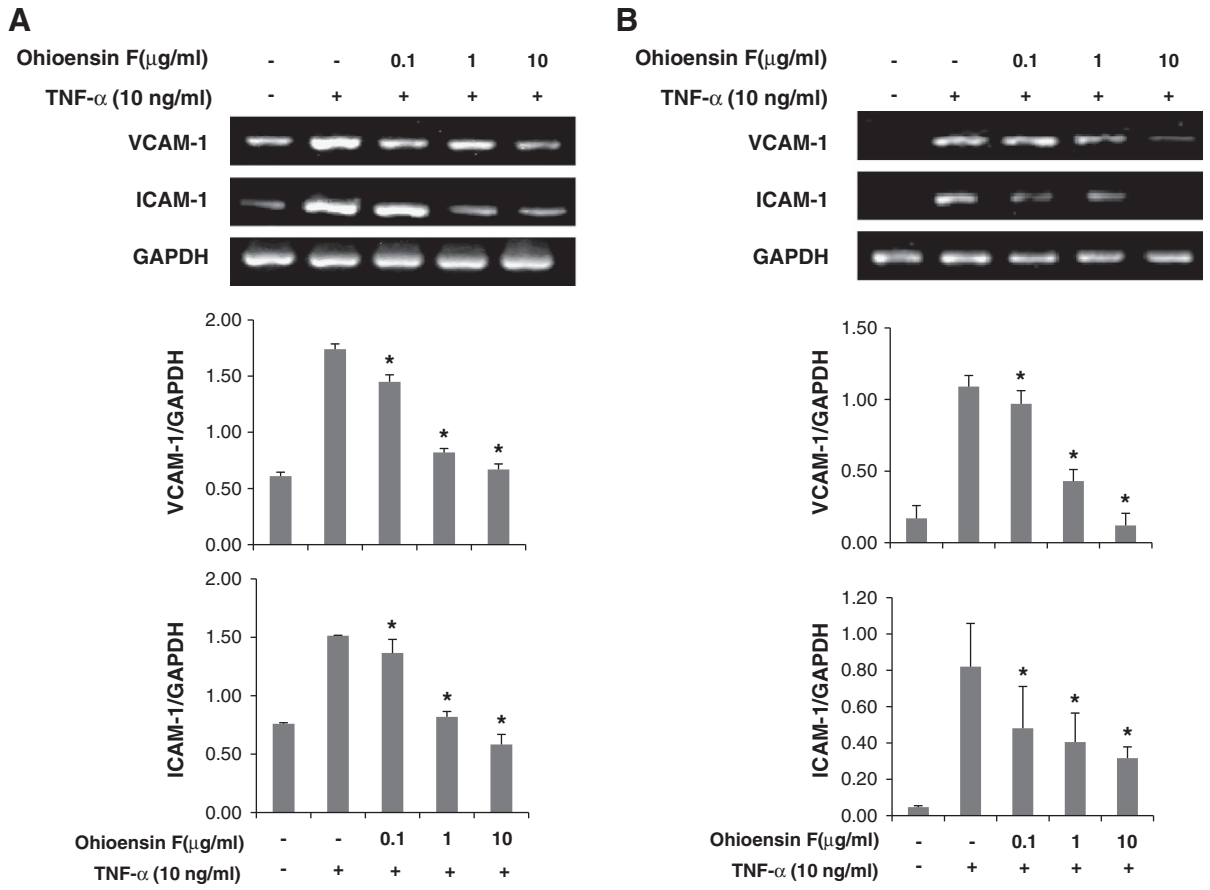

Fig. 4. Influence on TNF-α-induced mRNA expression of VCAM-1 and ICAM-1 by ohioensin F. MOVAS-1 cells (A) or HASMC (B) were pre-incubated with indicated concentrations of ohioensin F for 2 h and stimulated with TNF-α (10 ng/ml) for 4 h. Levels of the mRNA for adhesion molecules were determined by RT-PCR. GAPDH served as a housekeeping gene. Expression of VCAM-1 and ICAM-1 mRNA are in arbitrary units, and data are normalized to respective amount of GAPDH mRNA. Data are expressed as the mean ± S.E.M of 3 experiments. * Significantly different from TNF-α-stimulated cells not treated with ohioensin F ($P<0.05$).

cells and HASMC. These results suggest that the VCAM-1 and ICAM-1 expression induced by TNF-α is post-transcriptionally modulated by ohioensin F.

Inhibition of TNF-α-induced NF-κB activation by ohioensin F

It is well documented that NF-κB is ubiquitous transcription factor that plays a crucial role in adhesion molecule expression (Angel and Karin, 1991; Beg et al., 1993). We therefore examined the effect of ohioensin F on NF-κB transcriptional activation. The cells were treated with various concentrations of ohioensin F for 2 h before stimulation with TNF-α for 4 h. We used transcriptional activation assays to determine whether ohioensin F affects NF-κB dependent transcription. Stimulation by TNF-α resulted in an approximately 2.6-fold increase in luciferase activity, and this increase was significantly decreased by ohioensin F at 10 μg/ml (Fig. 5A). 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 ohioensin F on the expression of p65 NF-κB protein. As shown in Fig. 5B, pretreatment of VSMCs with ohioensin F dose-dependently reduced p65 NF-κB translocation to the nuclear fraction, suggesting that ohioensin F 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 (Pahl, 1999; Waddick and Uckun, 1999). To determine whether ohioensin F affects TNF-α-induced degradation of IκBα, we perform Western blot assay to detect the expression of IκBα protein in MOVAS-1 cells and HASMC (Fig. 5C and E). 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 both cells pre-incubated by ohioensin F TNF-α failed to degradation of IkBα. Consistent with the protein expression, a significant inhibitory effect of ohioensin F on the TNF-α-induced NF-κB p65 nuclear translocation determined by immunofluorescence assay was observed (Fig. 5D). These results further demonstrate that ohioensin F inhibits TNF-α-induced NF-κB activation.

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

Effect of ohioensin F on MAP kinases and Akt in TNF-α-stimulated 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 (Ju et al., 2002; Ho et al., 2008). The present data also showed that the ohioensin F treatment influenced vascular CAM expression. Therefore, the p38 MAPK, ERK1/2 and JNK kinase pathways were examined to determine if ohioensin F interferes with the expression of adhesion molecules by TNF-α. As shown in Fig. 6, the levels of activation of p38 MAPK, ERK1/2 and JNK in untreated cells were increased clearly by TNF-α treatment. However, the induced MAPK activity was significantly reduced by pretreatment with ohioensin F for 2 h.

Since Akt has been implicated in the regulation of adhesion molecule expression and NF-κB activation induced by TNF-α (Kang et al., 2006; Oh and Kwon, 2009), we further investigated the effect of

ohioensin F on TNF-α-induced Akt activation. Treatment of cells with ohioensin F resulted in the inhibition of TNF-α-induced Akt activation in a dose dependent manner (Fig. 6). These results suggest that ohioensin F may reduce TNF-α-induced VCAM-1 and ICAM-1 expression by reducing the activation of MAPK and Akt pathways.

Effects of ohioensin F on the production of ROS in TNF-α-stimulated smooth muscle cells

It has been suggested that TNF-α induced ROS production activates NF-κB in vascular cells (Yoon et al., 2010; Zhang and Wang, 2006). Our data also showed that treatment of cells with ohioensin F remarkably attenuated the activation of NF-κB. Therefore, the effect of ohioensin F on the production of TNF-α-induced ROS was investigated. VSMCs were pretreated with ohioensin F for 2 h and then stimulated with TNF-α. Ohioensin F significantly suppressed the production of TNF-α-induced ROS in a concentration-dependent manner (Fig. 7), implicating that ohioensin F inhibits NF-κB activation via the suppression of ROS production and has an antioxidant activity.

Discussion

TNF-α released by inflammatory cells is thought to play an important role in atherogenesis. Vascular smooth muscle cells have been known to participate in inflammatory responses in the vascular wall by expression of leukocyte adhesion molecules (Jang et al., 1994; Libby and Li, 1993). Vascular smooth muscle cells express the cellular adhesion molecules VCAM-1 and ICAM-1, which are inducible by

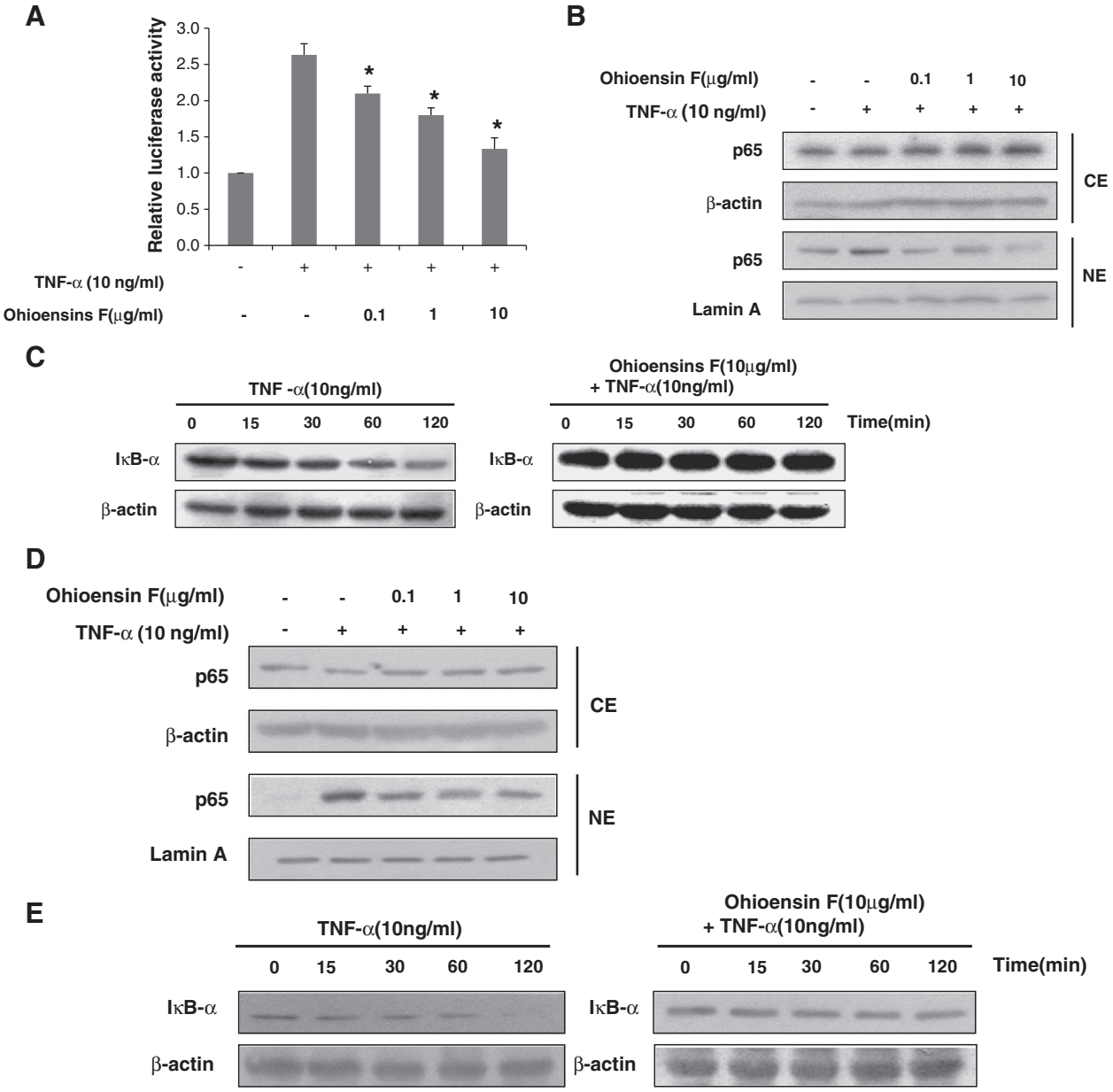

Fig. 5. Effects of ohioensin F on NF-κB activation/IκBα degradation and nuclear translocation of NF-κB/p65 in TNF-α-stimulated VSMCs. NE, nuclear extracts; CE, cytoplasmic extracts. (A) MOVAS-1 cells were transfected with a pGL3-NFκB-Luc reporter plasmid and pCMV-β-gal, pretreated with various concentrations of ohioensin F 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, C) or HASMC (D, E) were pre-incubated with or without various concentrations of ohioensin F 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. Lamin A and β-actin were used as loading controls for nuclear and cytosolic protein fractions, respectively. (F) Immunofluorescent imaging shows an ohioensin F-mediated suppression of TNF-α-mediated nuclear translocation of NF-κB p65 in MOVAS-1 cells. The arrow indicates the position of NF-κB p65 (magnification, ×200). Scale bars equal 200 μm.

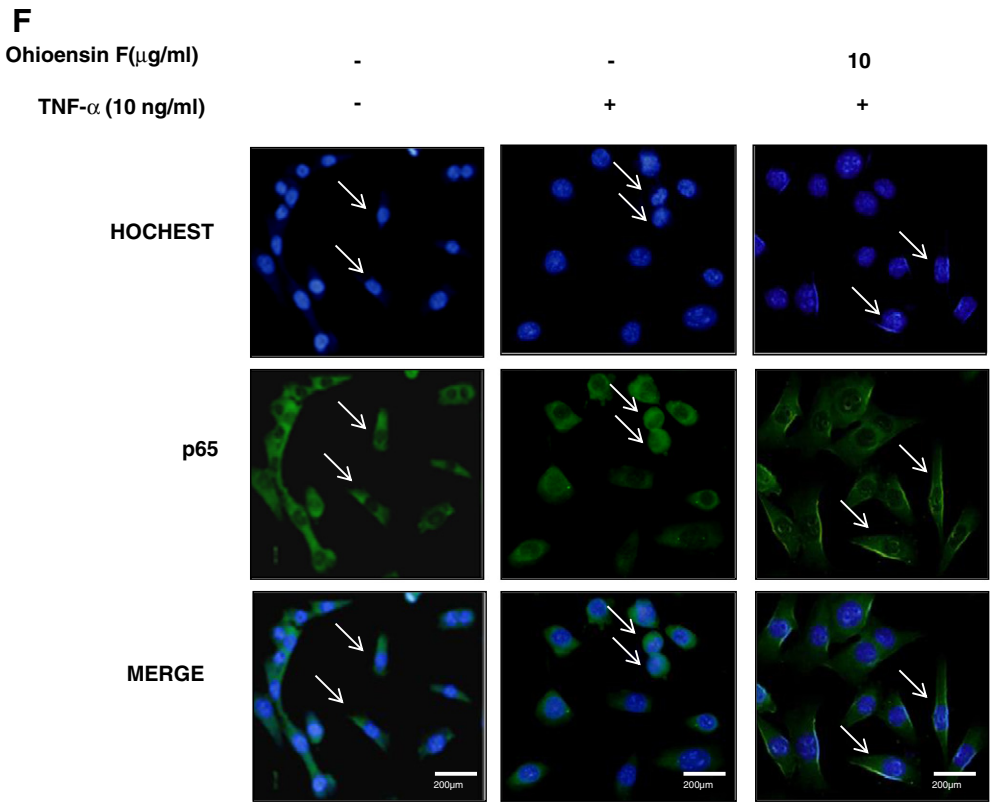

Fig. 5 (*continued*).

TNF-α in the atherosclerotic inflammatory disease. 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 (Kasper et al., 1996). Thus, pharmacological agents that inhibit the expression of these adhesion molecules have the potential to inhibit inflammatory diseases, such as atherosclerosis. In this study, our data demonstrate that ohioensin F obtained from Antarctic moss *P. alpinum*, has an anti-inflammatory activity. To our knowledge, this is the first report showing the inhibitory effect of ohioensin F on adhesion molecule expression in vascular smooth muscle cells.

In the present study, we evaluated the effects of ohioensin F on expression of the muscle adhesion molecule VCAM-1 and ICAM-1 as well as on monocyte adhesion to mouse and human vascular smooth muscle cells. Ohioensin F reduced induction of expression of adhesion molecules in a dose-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 ohioensin F. It has been known that the expression of adhesion molecules on VSMCs may facilitate the accumulation of transmigrated leukocytes within vascular wall (Libby and Li, 1993). 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 (Jang et al., 1994). Based on these findings, our data suggest that the changes in adhesion molecules by ohioensin F provide the vascular muscle cell with a mechanism of regulating cell–cell interactions during recruitment of monocytes.

It has been known that 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 (Ju et al., 2002; Ho et al., 2008). Many independent lines of evidences indicate 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 (Rao et al., 1995; Law et al., 1996; Hoshiya and Awazu, 1998). In addition, Akt has been known to be involved in the NF-κB activation and adhesion molecule expression induced by TNF-α (Kang et al., 2006; Oh and Kwon, 2009). Therefore, we have examined the effect of ohioensin F on Akt pathway and JNK, p38 MAPK and ERK1/2 pathways which are three well-characterized subtypes of MAPKs. Our data demonstrate that ohioensin F significantly inhibited phosphorylation of JNK, p38 MAPK, ERK1/2 and Akt phosphorylation in TNF-α-stimulated VSMCs. Thus, these results suggest that ohioensin F treatment inhibits TNF-α-induced adhesion molecule expression through inhibition of MAPK and Akt activation. As the present data demonstrated that ohioensin F 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 ohioensin F may inhibit the expression of the cell adhesion molecules by interfering with the transcription of their respective genes. It may suppress 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 (Collins et al., 1995). 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 (Pahl, 1999; Waddick and Uckun, 1999). In addition, it has been reported that NF-κB is essential to the gene expression of cell adhesion molecules, such as VCAM-1 (Ledebur and Parks, 1995). Therefore, the reduced expression of adhesion molecules prompted us to examine the effect of ohioensin F 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. Immunofluorescence assay showed that ohioensin F inhibited nuclear translocation of NF-κB p65. The present data also demonstrate that

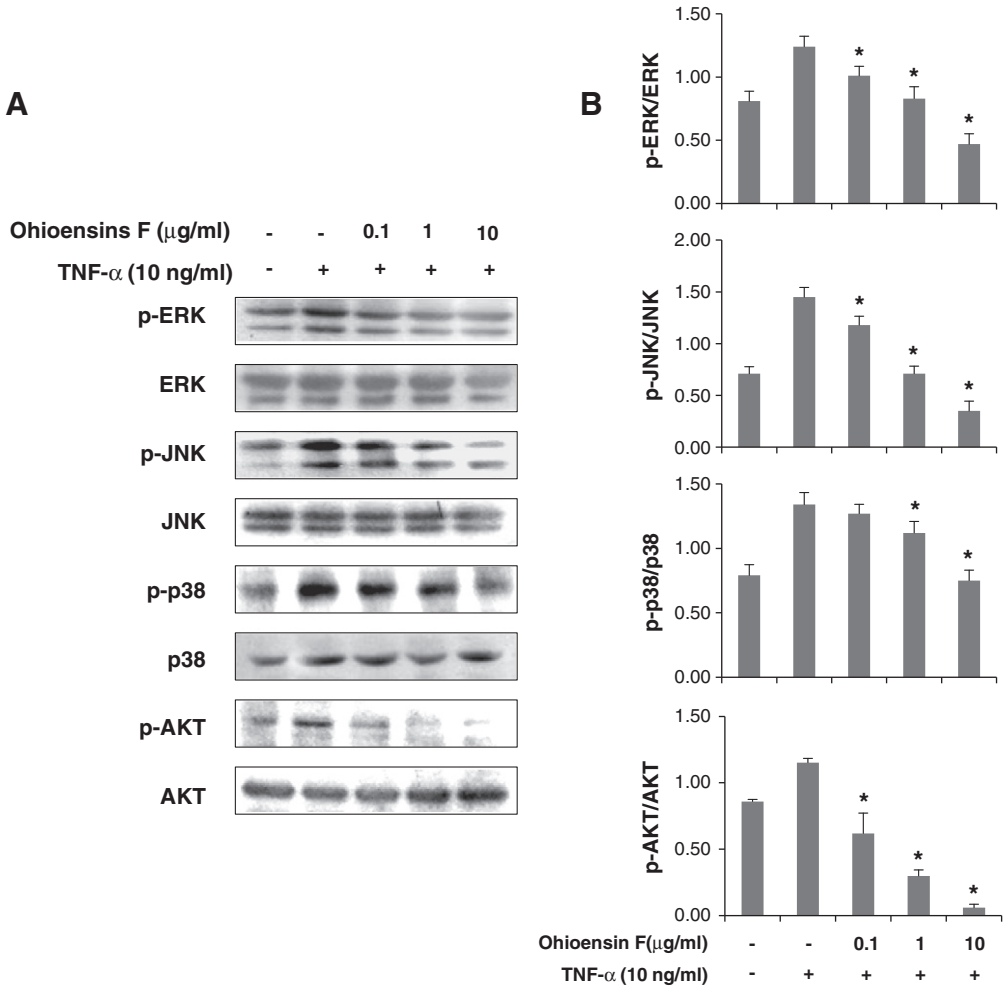

Fig. 6. Effect of ohioensin F on pp38, p-JNK, p-ERK and Akt activation in TNF-α-stimulated VSMCs. MOVAS-1 cells were pretreated with the indicated concentration of ohioensin F for 2 h and then incubated with TNF-α (10 ng/ml) for 30 min. The whole cell lysates were analyzed by Western blot. Immunoblot 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. The results are mean ± S.E.M of 3 experiments. *$p<0.05$, significantly different from the group treated with TNF-α.

ohioensin F 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 (Roebuck, 1999; Iademarco et al., 1992; Neish et al., 1995a,b). Therefore, the present data suggest that inhibitory effect of ohioensin F on adhesion molecule expression is at least partially mediated through suppression of the NF-κB transcription factor.

In consideration of the inflammatory nature of TNF-α exerting its cellular effect through receptors, it is important to examine the regulation of TNF receptor expression. Since it has been known that the numerous biological effects of TNF-α are signaled via TNFR1 that is the major signaling receptor in most cells (McCoy and Tansey, 2008), the initial signal of TNF-α in terms of the interaction of ligand with its receptor could be affected by ohioensin F. However, ohioensin F did not inhibit cell surface or total cellular protein expression of TNFR1 (data not shown), suggesting that the inhibitory effects of ohioensin F are not mediated by alterations in muscle cell surface TNFR1 expression. Nor can we formally rule out the possibility that ohioensin F could reduce TNFR1 activity by suppressing its nuclear localization, as in the case of the androgen receptor (Zhu et al., 2001). Moreover, TNF-α and its receptor have been suggested to activate the NF-κB signaling pathway and our data show that ohioensin F inhibited TNF-α-induced IKK activation and subsequent IκB degradation Based on these finding, our current results further indicate that the inhibition of adhesion molecule expression by ohioensin F seems to result from its specific inhibitory effects on NF-κB activation.

It has been suggested that many of the effects of cytokines on vascular cells could involve increases in reactive oxygen species (ROS) (Sprague and Khalil, 2009). Inflammatory cytokines like TNF-α may

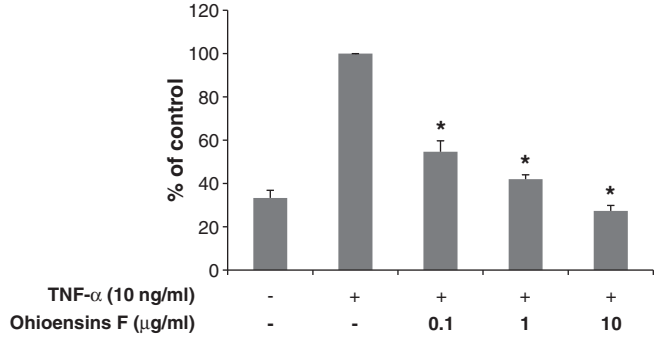

Fig. 7. Effects of ohioensin F on the production of ROS in TNF-α-stimulated VSMCs. MOVAS-1 cells were treated with ohioensin F at the concentrations indicated for 2 h followed by the stimulation with TNF-α (10 ng/ml) for 4 h. The level of ROS was measured as described in materials and methods. The results are mean ± S.E.M of quintuplicates from a representative experiment. *$p<0.05$, significantly different from the group treated with TNF-α.

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 (Stocker and Keaney, 2004). In the present study, pretreatment with ohioensin F significantly inhibited the TNF-α induced ROS production in vascular muscle cells, indicating that ohioensin F has similar 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 (Gosset et al., 1999; Gupta et al., 1999; Kunsh and Medford, 1999; Griendling et al., 2000; Guedes et al., 2008; Kim et al., 2008). Furthermore, adhesion molecule expression has been implicated to occur by stimulation of NF-κB signaling pathway through the induction of ROS (Qin et al., 2006). Therefore, it is plausible that the inhibitory effect of ohioensin F on the TNF-α induced expression of adhesion molecules and activation of NF-κB, Akt and MAPK is due to its antioxidant properties.

As the results described above, the results of the present study demonstrate that ohioensin F is capable of inhibiting the expression of VCAM-1 and ICAM-1 in VSMCs. Inhibition of cell adhesion molecule expression reduced adhesion of THP-1 cells to TNF-α-stimulated VSMCs, demonstrating pharmacological activity of ohioensin F in vascular muscle cells. The effect of ohioensin F is resulting from the suppression of ROS production, MAPK pathways, NF-κB and Akt activation. These data might account, at least in part, for the anti-inflammatory activities of ohioensin F.

In summary, ohioensin F prevents the expression of VCAM-1 and ICAM-1 and reduces monocyte adhesion to TNF-α-stimulated VSMCs. These results suggest a pharmacological activity of ohioensin F in vascular muscle cells. In addition, the effect of ohioensin F is resulting from the suppression of ROS production, MAPK pathways, NF-κB and Akt activation. Therefore, our finding that ohioensin F inhibits the expression of adhesion molecules may provide a new therapeutic strategy for the treatment of atherosclerosis.

Conflict of interest

All the authors declared no competing interests.

Acknowledgments

This work was supported by the Korea Polar Research Institute, grant number PE11060 (Utilization of novel metabolites from polar organiums).

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