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Ramalin inhibits VCAM-1 expression and adhesion of monocyte to vascular smooth muscle cells through MAPK and PADI4-dependent NF-kB and AP-1 pathways

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Cell adhesion molecules play a critical role in inflammatory processes and atherosclerosis. In this study, we investigated the effect of ramalin, a chemical compound from the Antarctic lichen *Ramalina terebrata*, on vascular cell adhesion molecule-1 (VCAM-1) expression induced by TNF-*α* in vascular smooth muscular cells (VSMCs). Pretreatment of VSMCs with ramalin (0.1–10 μg/mL) concentration-dependently inhibited TNF-*α*-induced VCAM-1 expression. Additionally, ramalin inhibited THP-1 (human acute monocytic leukemia cell line) cell adhesion to TNF-*α*-stimulated VSMCs. Ramalin suppressed TNF-*α*-induced production of reactive oxygen species (ROS), PADI4 expression, and phosphorylation of p38, ERK, and JNK. Moreover, ramalin inhibited TNF-*α*-induced translocation of NF-κB and AP-1. Inhibition of PADI4 expression by small interfering RNA or the PADI4-specific inhibitor markedly attenuated TNF-*α*-induced activation of NF-κB and AP-1 and VCAM-1 expression in VSMCs. Our study provides insight into the mechanisms underlying ramalin activity and suggests that ramalin may be a potential therapeutic agent to modulate inflammation within atherosclerosis.

Key words: ramalin; adhesion molecules; inflammation; transcription factor; PADI4

Atherosclerosis is a chronic, progressive, inflammatory disorder of the arterial wall that is characterized by an accumulation of extracellular lipid core in atheroma. Early in the development of atherosclerosis, inflammatory cells are recruited to vascular wall for their transendothelial migration.[1)] The accumulation of inflammatory cells in early step of atherosclerosis is the result of induced expression of proinflammatory adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), and vascular smooth muscle cell (VSMC) plays an important role in initiation of atherosclerosis.[2–4)] During the development of atherosclerotic lesions, VSMC interaction with inflammatory leukocytes contributes to further exacerbating the disease.[5)] Numerous studies have documented that VCAM-1 expression is highly induced in the neointimal VSMCs, which facilitates monocytes infiltration into atherosclerotic vascular wall and increases the proliferation of VSMC, implicating that VCAM-1 plays a dominant role in the initiation of atherosclerosis.[6–8)] In addition, it has been shown that activation of VSMCs by proinflammatory molecules including TNF-*α* increased the expression of VCAM-1.[9)] Based on these findings, a promising therapeutic approach for treating pathological inflammation is to reduce aberrant leukocyte adhesion to the VSMCs via suppression of adhesion molecules expression.

Recently, much attention has been given to the flora and fauna of the Antarctic. Additionally, interest in compounds derived from plant has been growing steadily over the past two decades because of their biological effects such as antioxidant and anti-inflammation.[10)] In the Antarctic region, lichens form the main part of the flora and many lichen species are found.[11)] Many lichens represent a source of natural products that have not been found elsewhere in nature and their natural products have been used in making dyes, cosmetics, as well as in medicines.[12)] In addition, they exhibit multiple biological activities, such as antibiotic, antimycobacterial, antiviral, anti-inflammatory, analgesic, antipyretic, antiproliferative, and cytotoxic effects.[13)] Thus, lichen substances could be the potential sources of pharmaceutically useful chemicals.

However, there is still large potential for further industrial screening and research on lichen products and their therapeutic potential remains pharmaceutically unexploited.

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Abbreviations: VCAM-1, vascular cell adhesion molecule 1; THP-1, human acute monocytic leukemia cell line; HRESIMS, high-resolution electrospray ionisation mass spectrometry; HMQC, heteronuclear multiple quantum correlation; HMBC, heteronuclear multiple bond correlation; PADI4, peptidyl arginine deiminase IV; MAPKs, mitogen-activated protein kinases; AP-1, activator protein 1; ROS, reactive oxygen species.

Ramalin isolated from the Antarctic lichen *Ramalina terebrata* has been suggested to be derived by condensation of γ-glutamyl-N′-(2-hydroxyphenyl) hydrazide [14,15] (Fig. 1). Recently, ramalin was demonstrated to have antioxidant and anti-inflammatory activities.[15] However, the basis of this effectiveness remains unknown. As the adhesion of monocytes to the vascular cells is a critical step in the inflammatory response, we hypothesized that ramalin might inhibit the expression of adhesion molecule induced by proinflammatory cytokine, TNF-α. To test our hypothesis, this study was designed to examine the effects and mechanisms of action of ramalin on the expression of VCAM-1 and adhesiveness of monocytes in VSMCs stimulated by TNF-α.

Materials and methods

Plant material. *Ramalina terebrata* was collected from the Korean Antarctic Research Station site on King George Island (S62° 13.3', W58°47.0'), Antarctica. The species was identified by Dr Soon Gyu Hong by comparing morphological characteristics with those previously published.[16] The voucher specimen was deposited in the Polar Lichen Herbarium, Korea Polar Research Institute, KOPRI, Incheon, South Korea.

Extraction and isolation. A freeze-dried and ground lichen sample (672 g) was extracted three times in mixture of methanol and water (5 L, 80:20, v/v). The resulting crude extract (83 g) was dissolved in 1 L of distilled water and sequentially partitioned three times with 1 L *n*-hexane and $CHCl_3$ to yield *n*-hexane (12.7 g), $CHCl_3$ (9.1 g), and water soluble (61.0 g) fractions. A portion (5 g) of the water soluble extract was then subjected to automated mild pressure liquid chromatography (MPLC) using a C_{18}ODS column (15 cm × 3 cm) and a stepwise gradient solvent system of 0, 20, 40, 60, 80, and 100% methanol in water. The fraction eluted at 0% methanol (2 g) was very active against DPPH free radical (IC50 = 8 μg/mL), and a portion (100 mg) of this fraction was subjected to semi-preparative reverse phase HPLC using a C_{18}ODS column (5 μm particle size, 250 mm × 10 mm). The gradient solvent system was 0% methanol in water (0.1% formic acid) over 10 min, 20% methanol over 20 min, and 100% methanol over 30 min. The flow rate was 2 mL/min. Compounds were detected by UV absorption at 280 nm. The fifth fraction (45 mg; tR = 18.88 min) was found to be most active against DPPH free radicals (IC50 = 1 μg/mL) and was therefore subjected to further purification by using repeated semipreparative HPLC using a C_{18}ODS column (250 mm × 10 mm). The gradient solvent system was 10–30% acetonitrile in water (0.1% formic acid) over 50 min with a flow rate of 2 mL/min. Ramalin (1, 30 mg) was eluted at 8.26 min. The molecular formula of ramalin [1,(- glutamyl-N′-(2-hydroxyphenyl) hydrazide, was determined as $C_{11}H_{15}N_3O_4$ by analysis of its HRESIMS (high-resolution electrospray ionisation mass spectrometry) data [m/z 254.1141 (M+H)+; (0.0 mmu)], indicating six degrees of unsaturation. This formula was supported by ^1H and ^{13}C NMR data.[15] The ^1H NMR spectrum indicated the presence of a methine, two methylenes, and four aromatic protons, requiring the presence of six exchangeable protons. In addition, ^{13}C NMR data showed the presence of two carbonyl and two quaternary sp^2 carbons (Table 1). Taken together, six degrees of unsaturation were accounted by the presence of two carbonyl and aromatic ring system. Analysis of HMQC (heteronuclear multiple quantum correlation) and HMBC (heteronuclear multiple bond correlation) data disclosed the partial carbon skeleton corresponding to the C-1–C-5 and 1, 2-disubstituted aromatic ring system.

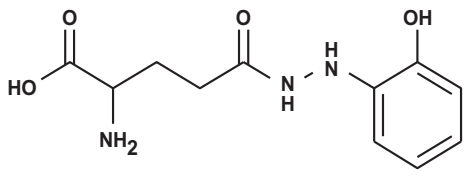

Fig. 1. Chemical structure of ramalin.

Reagents. Unless otherwise indicated, all chemicals used in this study were purchased from Sigma Chemical Co. (St Louis, MO). Anti-VCAM-1 antibodies were purchased from R & D Systems, USA. Metafectene PRO was purchased from Biontex (Martinsried, Germany). DMEM medium and fetal bovine serum (FBS) were purchased from Life Technologies, Inc. (Carlsbad, CA). pGL3-NF-κB, pGL3-AP-1, and the luciferase assay system were obtained from Promega (Madison, WI). pCMV-β-gal was obtained from Lonza

Table 1. ^1H and ^{13}C NMR spectroscopic data for ramalin in D_2O.

No.	δ_H[a] (int., mult., *J* in Hz)[α]	δ_C[b]	HMBC (H → C#)
1	—	165.3	
2	3.50 (1H, t, 6.2)	45.7	1, 3, 4
3	1.88 (2H, m)	17.7	1, 2, 4
4	2.22 (2H, m)	21.1	2, 3, 5
5	—	166.1	
1'	—	127.0	
2'	—	135.4	5
3'	6.60–6.53 (4H, m)	106.9[c]	
4'		112.7[c]	
5'		113.3[c]	
6'		105.3[c]	

[a]Recorded at 400 MHz.
[b]Recorded at 400 MHz.
[c]Assignments interchangeable.

(Walkersville, MD). 3-amino-1, 2, 4-triazole and N-α-benzoyl-N5-(2-chloro-1-iminoethyl)-L-Orn amide were purchased from Calbiochem (La Jolla, CA). Antibodies against PADI-4, IκB-α, p65, JNKs, phospho-JNK (p-JNK), ERK, phospho-ERK (p-ERK), phospho-p38 (-p38) c-jun, c-fos, PADI4, lamin A, and β-actin were purchased from Abcam Inc., USA.

Cell culture. The VSMC line MOVAS-1 was purchased from ATCC (Rockville, MD) and was grown in DMEM medium supplemented with 200 mg/mL G418, 100 IU/mL penicillin, 100 mg/mL streptomycin, and 10% heat-inactivated FBS in a humidified atmosphere containing 5% CO_2 at 37 °C. For subculture, the cells were detached using 0.125% trypsin containing 0.01 M EDTA. Cells used in this study were from first to sixth passage. Human aortic smooth muscle cells (HASMCs) were purchased from Clonetics Corp. (San Diego, CA). HASMCs 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. For subculture, 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 primary culture cells at passages five to eight. All experiments were carried out with the same batch of smooth muscle cells, which were from a single donor. THP-1 cells (ATCC), a human acute monocytic leukemia cell line, are widely used to study monocyte/macrophage biology in culture systems. THP-1 cells were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 100 μg/mL streptomycin, 100 IU/mL penicillin, and 10% FBS.

Measurement of cell viability. VSMCs were seeded at a concentration of 2×10^4 cells/well in 96-well tissue culture plates and treated with various concentrations of ramalin or 10 ng/mL TNF-α for the indicated times. Cell viability was measured by a quantitative colorimetric assay with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], which indicates the mitochondrial activity of living cells. The extent of 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). Cell viability was expressed as the percentage of the untreated control.

Cell adhesion assay. The method used for the adhesion assays is described elsewhere.[17] Briefly, VSMCs were grown in 96-well plates and pretreated with ramalin (0.1–10 μg/mL) for 2 h. The cells were washed with medium to remove ramalin and incubated with fresh growth medium containing TNF-α (10 ng/mL) for 6 h. The medium was removed from the wells and BCECF [2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein]-labeled THP-1 cells (2.5×10^5 cells/mL) in 0.2 mL medium were added to each well. The test and control samples were tested in triplicate 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 was determined by measuring the fluorescence intensity using a Cytofluor 2350 (Millipore, Bedford, MA). Increase in THP-1 cell adhesion upon stimulation of VSMCs with TNF-α was calculated relative to the basal adhesion of THP-1 cells to nonstimulated VSMCs, which was set to 100.

Determination of cell surface expression of adhesion molecules by ELISA. Cell surface expression of adhesion molecules on muscle cell monolayers was quantified by ELISA using a modification of the method described previously.[18] VSMCs were seeded at a concentration of 2×10^4 cells/well in 96-well gelatin-coated plates, cultured to confluence, and pretreated with ramalin (0.1–10 μg/mL) for 2 h at 37 °C. The cells were washed with medium to remove ramalin and then incubated with fresh growth medium containing TNF-α (10 ng/mL) for 8 h to determine the expression of VCAM-1. After incubation, the cells were washed with phosphate-buffered saline (PBS) pH 7.4 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 nonspecific binding. The cells were then incubated with monoclonal antibodies against 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 (1 mg/mL p-nitrophenyl phosphate in 0.1 M glycine buffer, pH 10.4, containing 1 m M $MgCl_2$, and 1 m M $ZnCl_2$). 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, and were subtracted from the experimental values.

Immunofluorescence. VSMCs were grown on 22-mm diameter glass coverslips and pretreated with ramalin (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, fixed with 3.7% formaldehyde in PBS for 15 min at room temperature, and washed again in PBS. Ice-cold methanol was added and the cells were incubated at −20 °C for 10 min, washed in PBS, and permeabilized with 1% BSA/0.2% Triton X-100/PBS for 1 h. The cells were washed in PBS, incubated with antibody against NF-κB p65 overnight at 4 °C, washed again, and incubated for 1 h with anti-rabbit IgG-FITC in 1% BSA/0.05% Triton X-100/PBS. Coverslips were mounted on glass slides using ProLong Gold antifade agent containing DAPI (Invitrogen) and photographed using a fluorescence microscope (BX51-Olympus Optical Co., Ltd, Ceneter Valley, PA). MOVAS-1 or HAMSCs with p65 localization were counted per 100 cells. Data were examined in a blind fashion by three independent reviewers totally unaware of all the culture conditions to prevent bias in their observation.

Measurement of mRNA levels by reverse transcription polymerase chain reaction (RT-PCR). 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 absorbance at 260 and 280 nm. PCR was performed using mouse VCAM-1-specific primers: sense primer, 5'-CCCAAGGATCCAGAGATTCA-3'; antisense primer, 5'-TAAGGTGAGGGTGGCATTTC-3'. The following human VCAM-1-specific primers were synthesized: sense primer, 5′- CATTTGACAGGCTG-GAGATA-3′ and antisense primer, 5′- GA-ACAGGTCATGGTCACAGA-3′. The PCR primers for the mouse GAPDH control were 5′-GGTCCTCAGTG-TAGCCCAAG-3′ (sense) and 5'-AATGTGTCCG-TCGTGGATCT-3′ (antisense). The following human GAPDH-specific primers were 5′-TCCCTCAA-GATTGTCAGCAA-3′ (sense) and 5′-AGATCCACA-ACGGATACATT-3′ (antisense). The absence of contaminants was routinely confirmed using negative control samples without primer addition. Samples were stored at -20 °C after amplification.

Transient transfection and reporter assays. Cells (5×10^5 cells/mL) were plated in 6-well plates and transiently co-transfected with the plasmids pGL3-NF-κB or pGL3-AP-1 and pCMV-*β*-gal using Metafectene PRO 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 Metafectene PRO reagent and added to the cells. For the luciferase assay, the cells were transfected with 0.5 μg AP-1 luciferase reporter using Metafectene PRO. After 4 h, the cells were pretreated with ramalin for 2 h followed by the addition of TNF-*α* for 4 h. Cells lysed with 200 μL lysis buffer (24 mM Tris–HCl (pH 7.8), 2 m M dithiothreitol, 2 m M EDTA, 10% glycerol, and 1% Triton X-100) and 10 μL of cell lysate were used for the luciferase activity assay. Luciferase and *β*-galactosidase activities were determined using the luciferase assay system (Promega). The values shown represent an average of three independent transfections, normalized to *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.[19] CMH_2-DCFDA (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 (3×10^5 cells/mL) were pretreated with various concentrations of ramalin for 2 h, followed by addition of TNF-α (10 ng/mL) for 4 h. The cells were stained for 15 min at 37 °C with 5 μ M CMH_2-DCFDA on ice in the dark. At least 10,000 cells for each sample were analyzed using a Becton Dickinson FACSCalibur (BD Biosciences, San Jose, CA). Changes in the level of intracellular ROS are expressed as a percentage of TNF-α-stimulated cells without ramalin treatment.

PADI4 transfection. To knock down PADI4 expression by siRNA, 100 nM of siRNA (Genolution Phamaceuticals,Seoul, Korea) was added to 500 μL of serum-free DMEM media without antibiotics, to which 6 μL of transfection reagent (Metafectene; Biontex, CA, USA) had been added and incubated for 15 min at room temperature. MOVAS-1 cells (5×10^5 cells/well) were cultured in 6-well plates overnight, washed once with serum-free media without antibiotics, and replaced with 1 mL of serum-free media containing antibiotics. Subsequently, the transfection reagent containing siPADI4 was added to the cell culture and incubated for 24 h. As a control, cells were transfected with mock siRNA. After 24 h incubation, siRNA was removed and replaced with new complete media and exposed to TNF-*α* (10 ng/mL) for 4 h followed by washing and lysis.

Western blot analysis. Western blot analysis was performed as previously described.[20] The cells were pretreated with ramalin (0.1–10 μg/mL) for 2 h. The cells were washed with medium to remove ramalin 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 phosphate-buffered saline (PBS) and suspended in 70 μL of buffer A [10 m M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.9), 1.5 m M $MgCl_2$, 10 m M KCl, 0.5 m M DTT, 0.5 m M phenylmethylsulfonyl fluoride (PMSF), and Protease Inhibitor Cocktail (Sigma)] and incubated on ice. After 15 min, 0.5% Nonidet P-40 was added to lyse the cells, followed by vortexing for 10 s. Cytosolic cell extracts were obtained after centrifugation at 1,500 × *g* for 10 min at 4 °C. The collected nuclei were resuspended in 50 μL of buffer C [20 m M HEPES (pH 7.9), 1.5 m M $MgCl_2$, 420 m M MNaCl, 0.2 m M EDTA, 25% *v/v* glycerol, 0.5 m M 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. Whole cell lysates and cytosolic and nuclear extracts (20 μg) were resolved on a 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel, electrophoretically transferred to an immobilon polyvinylidene difluoride membrane (Amersham, Arlington Heights, 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 protein loading.

Statistical analysis. Each result is reported as means ± SEM. One-way analysis of variance was used to determine significance among groups, after which the modified *t* –test was performed.

Results

Effect of ramalin on cell viability in VSMCs

We examined the effect of ramalin on viability of VSMCs using the MTT assay at the indicated

concentrations of ramalin or TNF-α for 8 h. The cytotoxicity of ramalin or TNF-α was not significantly observed in MOVAS-1 cells and HASMCs. Additionally, ramalin and TNF-α at concentrations used in the experiments did not affect cell viability as assessed by trypan blue staining and morphology of VSMCs, but concentrations > 10 μg/mL were found to be cytotoxic (data not shown).

Effect of ramalin on VCAM-1 expression and adhesion of monocyte to smooth muscle cells

We investigated the effect of different ramalin concentrations on the adherence of leukocytes to TNF-α-activated VSMCs. TNF-α treatment resulted in an increased adhesive capacity of smooth muscle cells through induction of adhesion molecule expression. As shown in Fig. 2(A), treatment of confluent MOVAS-1 with 10 ng/mL TNF-α for 8 h resulted in about 2.5-fold increase in adhesion of THP-1 monocytic cells compared with adhesion of THP-1 cells to untreated VSMCs. In a concentration-dependent manner, pretreatment of the cells for 2 h with ramalin (0.1–10 μg/mL) decreased the binding of monocytes to VSMCs. Moreover, similar findings were observed using an additional set of primary human cell, HASMC (Fig. 2(B)). TNF-α-induced adhesion of THP-1 cells to HASMC was dose-dependently reduced by preincubation with increasing concentrations of ramalin for 2 h prior to TNF-α treatment. To examine the mechanism whereby ramalin inhibited the binding of monocyte to VSMCs, we investigated VCAM-1 expression, which is known to support the binding of monocytes.[8] MOVAS-1 cells and HASMC were preincubated without or with various concentrations of ramalin for 2 h and then stimulated with TNF-α (10 ng/mL) for 8 h. As detected by ELISA, exposure of cells to TNF-α induced strong upregulation of surface expression of VCAM-1. However, ramalin pretreatment significantly inhibited in the TNF-α-induced VCAM-1 expression in a concentration-dependent manner (Fig. 3(A)). In addition, to compare the possibility that total cellular adhesion molecule differs from that only expressed at the cell surface, we examined the expression of total cellular adhesion molecule on VSMCs in response to TNF-α stimulation and ramalin treatment. Ramalin significantly suppressed TNF-α induced VCAM-1 protein level in a concentration-dependent manner in both cell types (Fig. 3(B) and (C)). The inhibitory effect of ramalin was selective, because ramalin did not affect the expression of β-actin. Taken together, our results suggest that ramalin is effective in inhibiting the induced level of VCAM-1 expression.

The experiments described above demonstrated that ramalin significantly inhibited the VCAM-1 in TNF-α-stimulated VSMCs. It was possible that ramalin inhibited VCAM-1 expression by regulating 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-specific probe. VSMCs were pretreated with ramalin (0.1–10 μg/mL) for 2 h, and then stimulated with TNF-α for 4 h. As shown in Fig. 3(D) and (E), the mRNA level of VCAM-1 was dose-dependently decreased with ramalin in MOVAS-1 cells and HASMC, suggesting that ramalin post-transcriptionally modulates TNF-α-induced VCAM-1 expression.

Inhibitory effect of ramalin on TNF-α-induced activation of NF-κB and AP-1

Since VCAM-1 promoter includes binding sites for NF-κB and AP-1[21,22] and these transcription factors have been known to play role as important mediators of adhesion molecule expression,[23,24] we investigated the effect of ramalin on TNF-α-induced activation of NF-κB and AP-1. The cells were preincubated with a variety of concentrations of ramalin for 2 h prior to stimulation with TNF-α for 4 h. We measured transcriptional activity to determine whether ramalin regulates NF-κB and/or AP-1 dependent transcription levels in TNF-α-stimulated MOVAS-1 cells. As shown in Fig. 4(A), stimulation of the cells with TNF-α resulted in an approximately 2.8-fold increase in luciferase activity, and the increased activity was significantly reduced by ramalin in a concentration-dependent manner. By western blot analysis, we also observed a significant reduction in the expression nuclear p65 NF-κB protein that regulates transcription of genes that control inflammation as compared to untreated cells (Fig. 4(B) and (D)). These results suggest that ramalin exposure resulted in inhibition of the TNF-α-induced nuclear translocation of NF-κB.

To further clarify the mechanism of NF-κB inhibition by ramalin, we examined the effect of this compound on degradation of IκBα which is involved in the translocation of NF-κB to the nucleus. Analysis of cell extracts using IκBα-specific antibody showed that significant degradation of IκBα was seen after 15 min by stimulation with TNF-α, whereas ramalin treatment resulted in the prevention of TNF-α-induced IkBα degradation in both cell types (Fig. 4(C) and (E)). Consistent with the protein expression, a significant inhibitory effect of ramalin on the TNF-α-induced NF-κB p65 nuclear translocation determined by immunofluorescence assay was observed in both cell types (Fig. 5(A) and (B)). These results further demonstrate that ramalin inhibits TNF-α-induced NF-κB activation in MOVAS-1 cells and HASMC.

We next determined whether ramalin can affect the activation of another transcription factor, AP-1. MOVAS-1 cells were transiently co-transfected with a plasmid mixture of the AP-1-luciferase reporter gene and the pcDNA3.1 blank vector. AP-1 dependent transcriptional activity of the reporter gene construct was enhanced by TNF-α, but ramalin significantly suppressed TNF-α-induced AP-1 activity in a concentration-dependent manner (Fig. 6(A)). The results also showed that ramalin treatment decreased the level of nuclear c-Jun and c-Fos proteins, two AP-1 components, in MOVAS-1 cells (Fig. 6(B)). Moreover, similar results were confirmed in another smooth cell line, HASMC (Fig. 6(C)). Thus, our results suggest that ramalin inhibits the TNF-α-induced nuclear translocation of AP-1. Collectively, our results suggest that ramalin inhibits the activation of NF-κB and AP-1, which might be associated with the blocking of TNF-α-induced VCAM-1 production by ramalin.

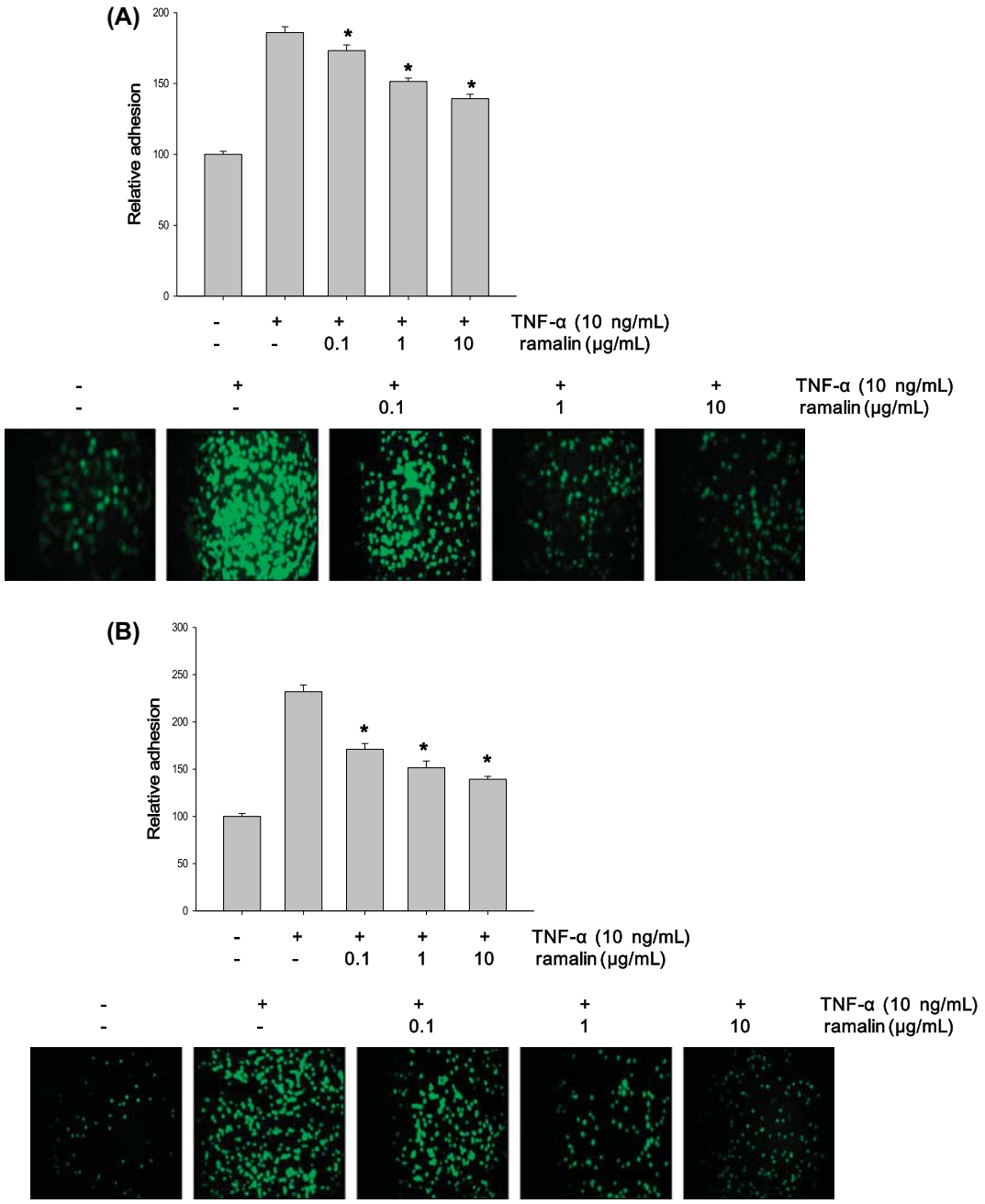

Fig. 2. Ramalin inhibits adhesion of inhibition of THP-1 cells to TNF-α-stimulated VSMCs.

Notes: The BCECF-labeled THP-1 cells were added to TNF-α (10 ng/mL)-stimulated MOVAS-1 cells (A) or HASMC (B) with or without ramalin pretreatment. The adhesion was measured as described in materials and methods. Adherent THP-1 to VSMCs was observed under a fluorescent microscope at 100× magnification. The results are expressed as the mean ± SEM of three independent experiments performed in triplicates. *Significantly different from TNF-α-stimulated cells not treated with ramalin ($p < 0.05$).

Effect of ramalin on MAP kinases (MAPKs) in TNF-α-stimulated smooth muscle cells

In our previous study, we found that the MAPK was involved in TNF-α-induced VCAM-1 expression in VSMCs.[25] In addition, the present study demonstrates that ramalin influence VCAM-1 expression. Therefore, the p38 MAPK, ERK1/2, and JNK kinase pathways were examined to determine if the inhibitory effect of ramalin on the expression of VCAM-1 is dependent on the MAPK pathways in TNF-α-stimulated smooth muscle cells. As shown in Fig. 7(A) and (B), treatment of TNF-α significantly resulted in an increase in the levels of activation of p38 MAPK in both cell types, ERK1/2 and JNK, whereas the induced MAPK activity was remarkably inhibited by pretreatment with ramalin for 2 h. These data suggest that ramalin may inhibit TNF-α-induced VCAM-1 expression by decreasing the activation of MAPK.

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

Considering that TNF-α-induced ROS production activates NF-κB and AP-1 pathways in various cells [26–28] and ramalin treatment significantly inhibits the activation of NF-κB and AP-1, we determine the effect of Ramalin on the production of TNF-α-induced ROS. VSMCs were pretreated with ramalin for 2 h and then

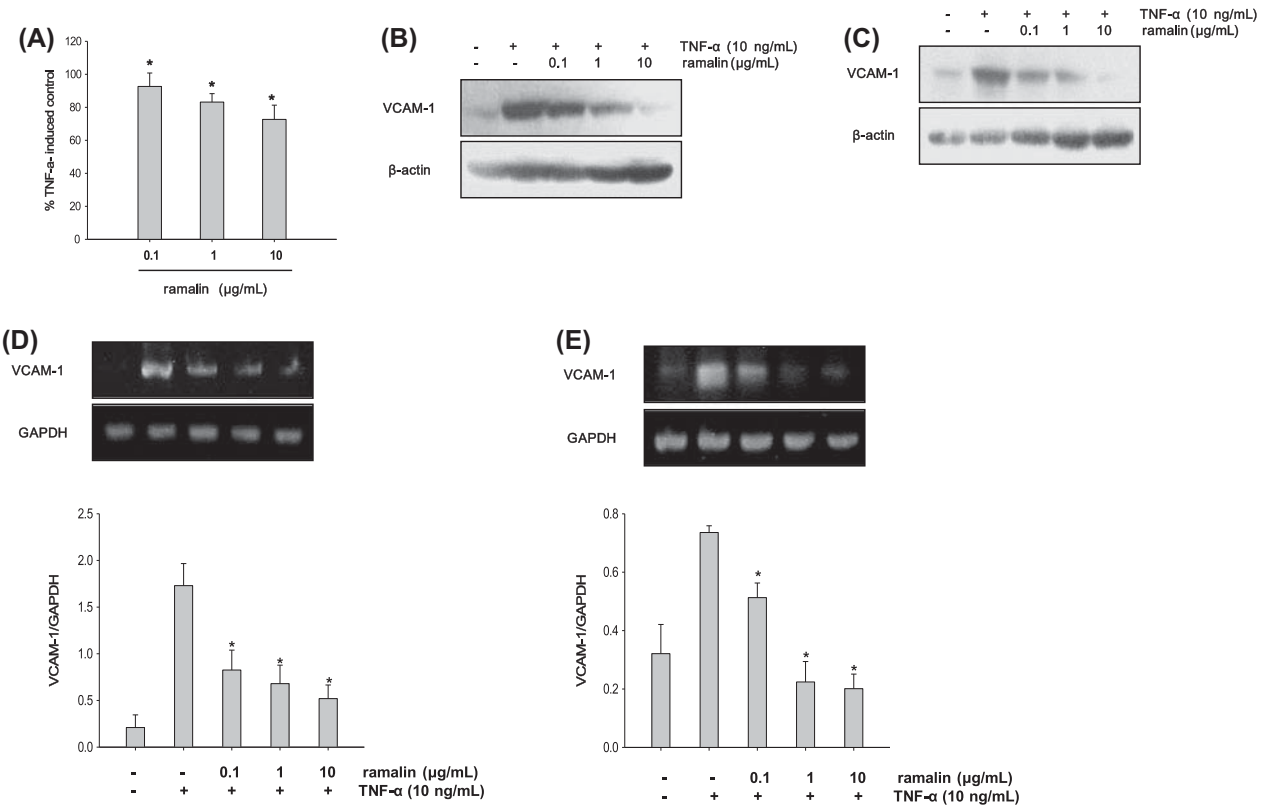

Fig. 3. Ramalin inhibits TNF-α-enhanced expression of VCAM-1 protein and mRNA.

Notes: (A) Expression of VCAM-1 in MOVAS-1 cells after preincubation with the indicated concentrations of ramalin for 2 h and stimulated with TNF-α (10 ng/mL) for 8 h was measured by ELISA. The data are expressed as a percentage of TNF-α-induced adhesion molecule expression. The VCAM-1 protein levels in MOVAS-1 cells (B) and HASMC (C) were determined by western blot assay. The β-actin protein level was considered as an internal control. MOVAS-1 cells (D) and HASMC (E) were preincubated with indicated concentrations of ramalin 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 intensity of the bands was quantitated by densitometry ($n = 3$). Expression of VCAM-1 mRNA is in arbitrary units, and data are normalized to respective amount of GAPDH mRNA. The results illustrated are from a single experiment, as a representative of three separate experiments. The intensity of the bands was quantitated by densitometry. The levels of VCAM-1 expression are in arbitrary units, and data are normalized to respective amount of GAPDH mRNA. Data are expressed as the mean ± SEM of 3 experiments. *Significantly different from TNF-α-stimulated cells not treated with ramalin ($p < 0.05$).

stimulated with TNF-α for 6 h. As shown in Fig. 8(A) and (B), ramalin significantly inhibited the production of TNF-α-induced ROS in a concentration-dependent manner in both cells, indicating that ramalin has an antioxidant activity. Next, we examined whether ROS mediate TNF-α-induced expression of VCAM-1, VSMCs were pretreated with N-acetyl cysteine (NAC), a well-known antioxidant, for 2 h before TNF-α-stimulation. NAC significantly inhibited the expression of VCAM-1 induced by treatment with TNF-α (Fig. 8(C) and (D)). Taken together, these and other observations suggest that ramalin could inhibit TNF-α-mediated NF-κB activation and subsequent VCAM-1 expression by preventing ROS generation.

Effect of ramalin on TNF-α-induced PADI4 expression

It has been reported that PADI4, a calcium dependent enzyme, plays a role in gene regulation and inflammatory diseases.[29–30] It is possible that ramalin might affect the level of PADI4 expression. To test this possibility, we examined the effect of ramalin on the level of PADI4 protein expression in TNF-α-treated VSMCs and results are shown in Fig. 9. PADI4 production was significantly increased by TNF-α, whereas pretreatment of VSMCs with ramalin significantly reduced the level of PADI4 in a concentration-dependent manner (Fig. 9(A) and (B)). Our data also shows that NAC significantly inhibited the expression of PADI4 induced by treatment with TNF-α in both cell types (Fig. 9(C) and (D)), implicating that ROS is an upstream regulator of PADI4 in TNF-α-stimulated VSMCs.

To determine whether PADI4 is involved in the VCAM-1 expression and NF-κB and AP-1 activity in TNF-α-treated VSMCs, PADI4-specific inhibitor, Cl-amidine (N-α-benzoyl-N^5-(2-chloro-1-iminoethyl)-L-ornithine amide),[31] and PADI4-specific siRNA were used to specifically reduce endogenous PADI4 expression. It was observed that Cl-amidine or siRNA-PADI4 suppressed TNF-α-induced VCAM-1 expression in MOVAS-1 cells (Fig. 10(A) and (B)). In addition, treatment with Cl-amidine inhibits NF-κB and AP-1 activity. This inhibition of PADI4 also decreased p65, c-Jun, and c-Fos expression. Thus, these results indicate that PADI4 is required for VCAM-1 expression and the activation of NF-κB and AP-1 in TNF-α-treated VSMC (Fig. 10(C)). Furthermore, the present data showed that

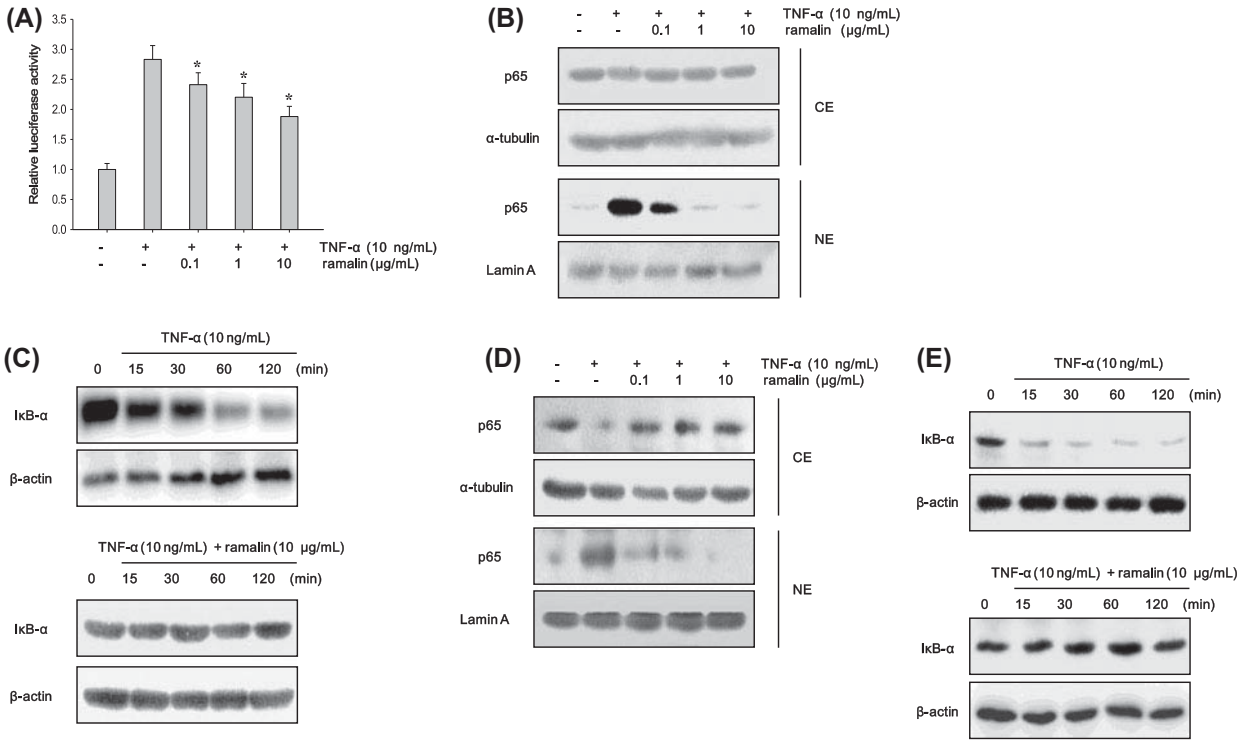

Fig. 4. Effects of ramalin on NF-κB activation and IκBα degradation in TNF-α-stimulated VSMCs. NE, nuclear extracts; CE, cytoplasmic extracts.

Notes: (A) MOVAS-1 cells were transfected with a pGL3-NFκB-Luc reporter plasmid and pCMV-β-gal, pretreated with various concentrations of ramalin for 2 h, and then stimulated with TNF-α (10 ng/mL) for 4 h. MOVAS-1 cells (B, C) and HASMC (D, E) were preincubated with or without various concentrations of ramalin for 2 h and 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. α-Tubulin and Lamin A were used as loading controls for cytosolic and nuclear protein fractions, respectively. The results illustrated are from a single experiment, as a representative of three separate experiments.

pretreatment of VSMCs with MAPKs inhibitors for 2 h before TNF-α exposure displayed inhibitory effects on the up-regulated PADI4 level by TNF-α (Fig. 10(D)). This implicates that MAPK works well as an upstream regulator of PADI4 expression. Taken together, results of this study suggest that ramalin inhibits PADI4-mediated activation of NF-κB and AP-1, which leads to inhibition of VCAM-1 in TNF-α-treated VSMCs.

Discussion

In this study, we demonstrate that pretreatment with ramalin obtained from the Antarctic lichen *Ramalina terebrata* inhibited the TNF-α-stimulated VCAM-1 expression. In addition, ramalin treatment inhibited the binding of the human monocytic cell line, THP-1, to TNF-α-stimulated VSMCs. This inhibitory effect of ramalin was mediated, at least in part, by blockage of the MAPK and PADI4-dependent NF-κB and AP-1 signaling pathways via the induction of ROS.

Ramalin has been known to have antioxidant and anti-inflammatory activities.[15] However, the precise mechanism by which ramalin exerts its beneficial effects remains unknown. The possible inhibition by ramalin of adhesion molecule expression could play a critical role in the prevention or treatment of inflammatory diseases, such as atherosclerosis. In the present study, we examined the effects of ramalin on VCAM-1 expression as well as on monocyte adhesion to VSMCs. We found that ramalin attenuated VCAM-1 expression in a dose-dependent manner after stimulation with TNF-α at the level of protein and mRNA. In corroboration of these results, ramalin had significant inhibitory effect on THP-1 cell adhesion to VSMCs, indicating the functional relevance of decreased VCAM-1 expression. VCAM-1 expression on VSMCs has been suggested to facilitate the accumulation of transmigrated leukocytes into vascular wall.[7] Interactions of VSMCs with monocytes via cell adhesion molecules might be crucial in the progression of atherosclerotic plaques.[32] Based on these findings, our results suggest that the inhibition of VCAM-1 expression by ramalin provide the VSMC with a mechanism of regulating cell–cell interactions during monocyte recruitment.

Several studies have shown that MAPKs are involved in the regulation of cell adhesion molecules expressed on cells in response to external stimuli including TNF-α.[18,33] It was also reported that cells may utilize different MAPK signaling pathways for TNF-α-induced expression of adhesion molecules and that the MAPK signaling pathways differently depend on anti-inflammatory compounds.[33–36] Therefore, in the present study, we examined the effect of ramalin on JNK, p38 MAPK, and ERK 1/2 pathways which are three well-characterized subtypes of MAPKs. We observed that ramalin significantly blocked phosphorylation of JNK, p38 MAPK, and ERK 1/2 in TNF-α-stimulated VSMCs, suggesting that ramalin treatment

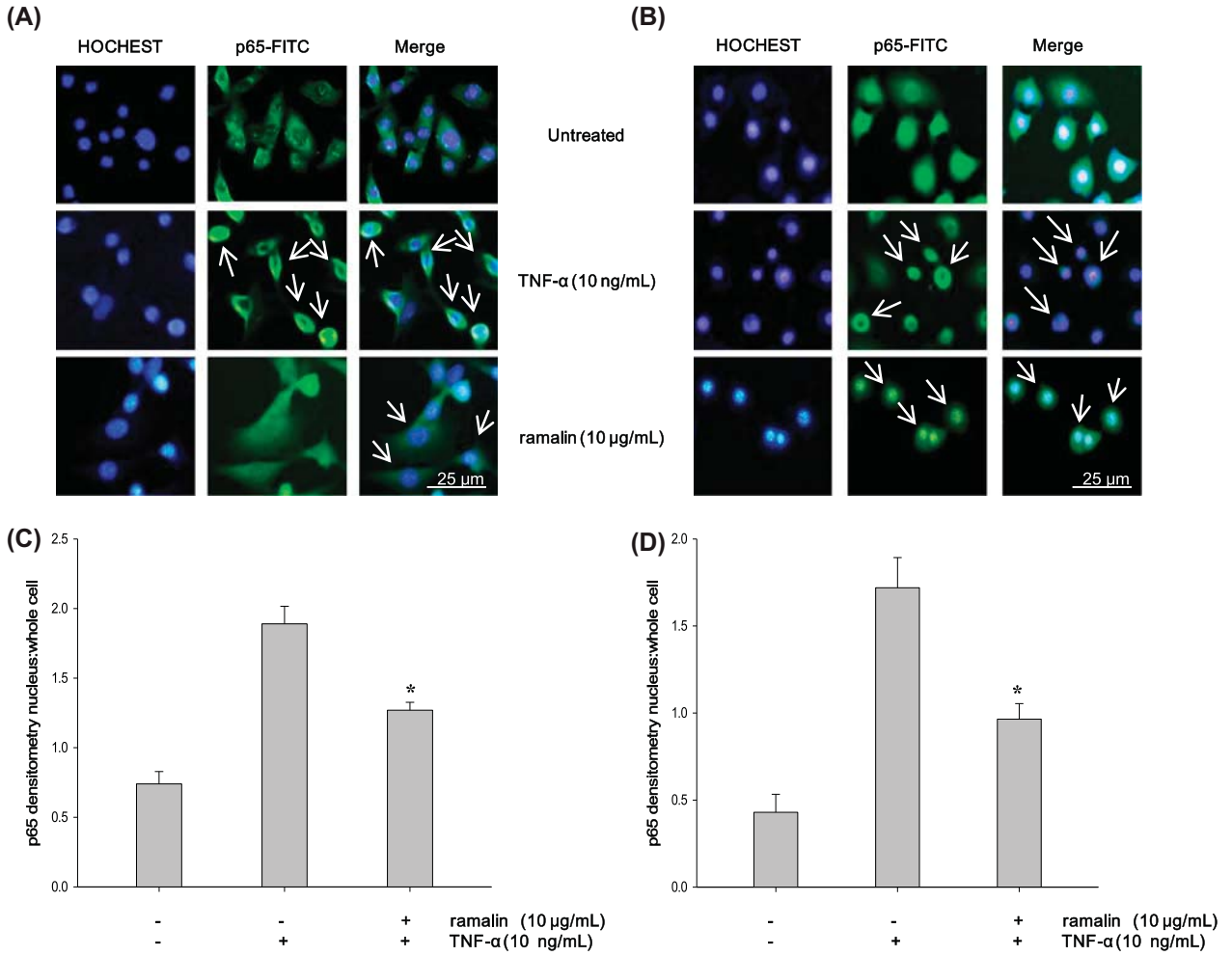

Fig. 5. Effects of ramalin on p65 gene translocation in TNF-α-stimulated VSMCs.

Notes: Confluent MOVAS-1 cells(A) or HASMCs(B) were pretreated with ramalin (10 μg/mL) for 2 h. The cells were washed twice with medium and incubated with TNF-α (10 ng/mL) for 4 h. The p65-FITC was added to the VSMCs for 12 h and HOCHEST was added to VSMCs for 20 min prior to observation. The p65-FITC translocation from cytosol to nucleus was observed under a fluorescent microscope at 400 x magnification. The results illustrated are representative of three separate experiments. Number of p65 (green) translocation to nucleus in MOVAS-1 cell (C) of HASMCs (D) was counted and results expressed in graph bars as the mean ± SEM, $n = 3$ per groups. *Significantly different from the group treated with TNF-α. ($p < 0.05$).

inhibits TNF-α-induced VCAM-1 expression through inhibition of MAPK activation.

Previous reports demonstrated that the activation of MAPK leads to activation of transcription factors which are important molecular targets for pharmacological intervention and drug development, and activation of transcription factors NF-κB and AP-1 by TNF-α is required for the transcriptional activation of cell adhesion molecules, including VCAM-1 and ICAM-1.[23,25,37] NF-κB activation was associated with the phosphorylation and degradation of IκBα, and the nuclear translocation of p65, which promotes the expression of downstream genes, such as VCAM-1. Therefore, we examined the effect of ramalin on NF-κB activation. We found that ramalin inhibited TNF-α-induced NF-κB activation through inhibition of IκB kinase activation and subsequent IκBα degradation. Furthermore, ramalin significantly attenuated TNF-α-induced AP-1 activity, and c-Jun and c-Fos protein levels in VSMCs, suggesting that reduced AP-1 activity in ramalin-treated cells may involve, at least in part, the downregulation of c-Jun and c-Fos expression. Thus, this mechanism appears to be involved in the action of ramalin on VCAM-1 expression in TNF-α-treated VSMCs. Our results do not totally rule out the possibility that TNF-α-induced VCAM-1 expression is mediated via other transcription factors and signaling pathways, because IRF-1, SP-1, and Nrf2 are known to be involved in gene expression of adhesion molecules.[38–40] However, our data implicating MAPK, NF-κB, and AP-1 pathways do represent a novel insight into the mechanism of ramalin effects on VCAM-1 expression in VSMCs.

It has been known that intracellular ROS play a critical role in the regulation of cell adhesion and ROS production induced by TNF-α leads to the enhanced expression of adhesion molecules.[41,42] Additionally, ROS has been implicated in the pathogenesis of atherosclerosis.[43] In the present study, pretreatment with ramalin significantly reduced the TNF-α-induced ROS production in VSMCs, indicating that ramalin has antioxidant activity. It was reported that ROS activates various transcription factors in cultured VSMCs and may function as a signaling molecule in various pathways leading to MAPK, NF-κB, and AP-1 activation.[42,44–48]. Furthermore, ROS has been implicated to

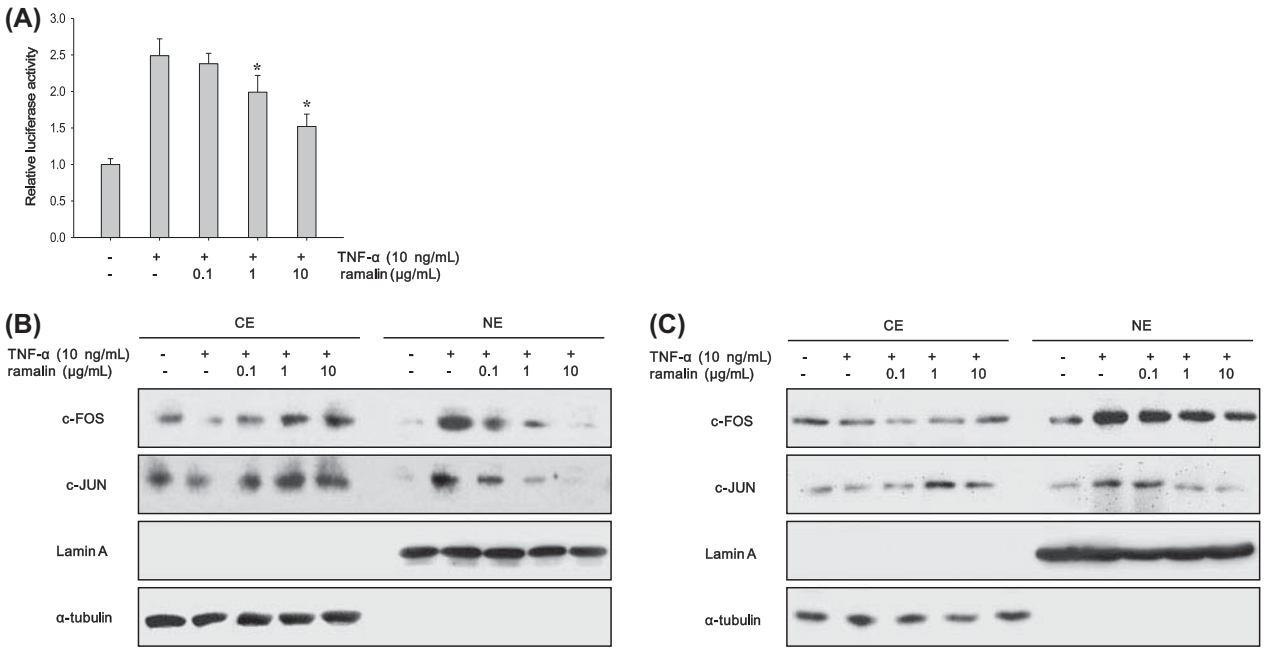

Fig. 6. Effects of ramalin on AP-1 activation, and c-Jun and c-Fos expression in TNF-α-stimulated VSMCs.

Notes: NE, nuclear extracts; CE, cytoplasmic extracts. (A) MOVAS-1 cells were transfected with a pGL3–AP-1–Luc reporter plasmid and pCMV-β-gal, pretreated with various concentrations of ramalin for 2 h followed by TNF-α treatment for 4 h. MOVAS-1 cells (B) and HASMCs (C) were preincubated with or without various concentrations of ramalin for 2 h and then treated with TNF-α (10 ng/mL) for 4 h. The protein levels of c-Jun and c-Fos were detected by western blotting to analyze the translocation of AP-1. Lamin A and α-tubulin were used as loading controls for nuclear and cytosolic protein fractions, respectively. The results illustrated are from a single experiment, as a representative of three separate experiments.

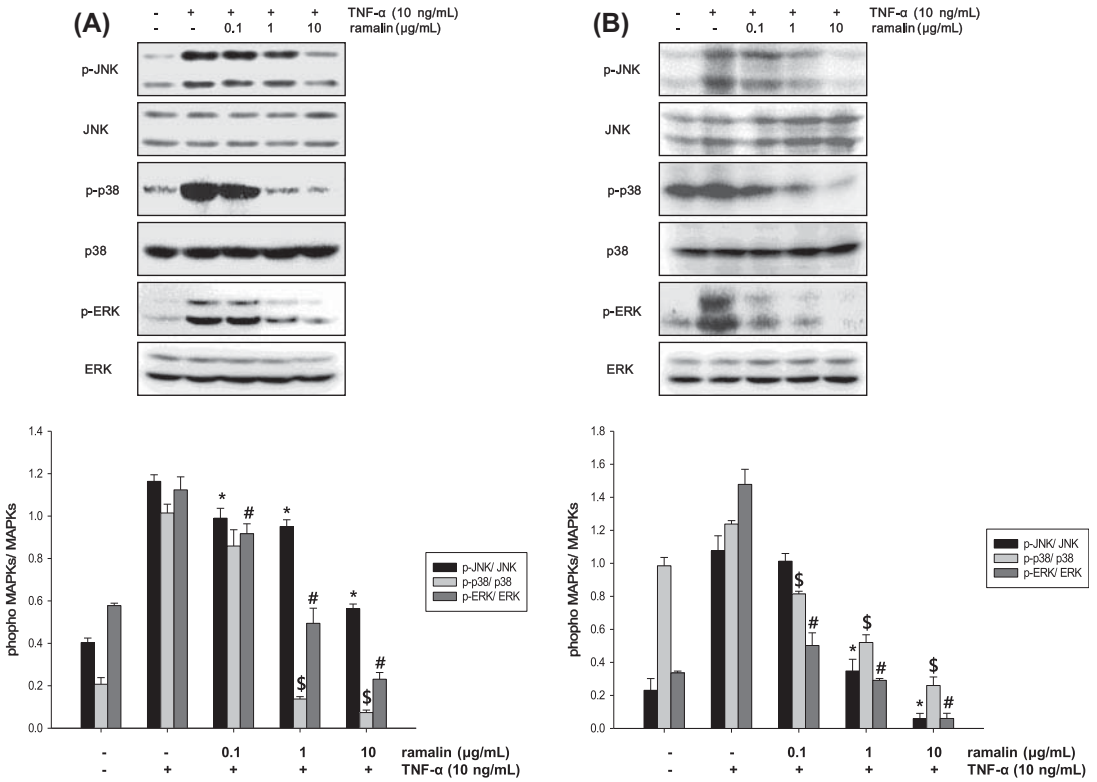

Fig. 7. Effect of ramalin on activation of MAPKs in TNF-α-stimulated VSMCs.

Notes: MOVAS-1 cells (A) and HASMCs (B) were pretreated with the indicated concentration of ramalin 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 ($n = 3$). The relative intensities were expressed as the ratio of phospho-MAPK to total MAPK. A typical result from three independent experiments is shown. *, $, #. $p < 0.05$, significantly different from the group treated with TNF-α.

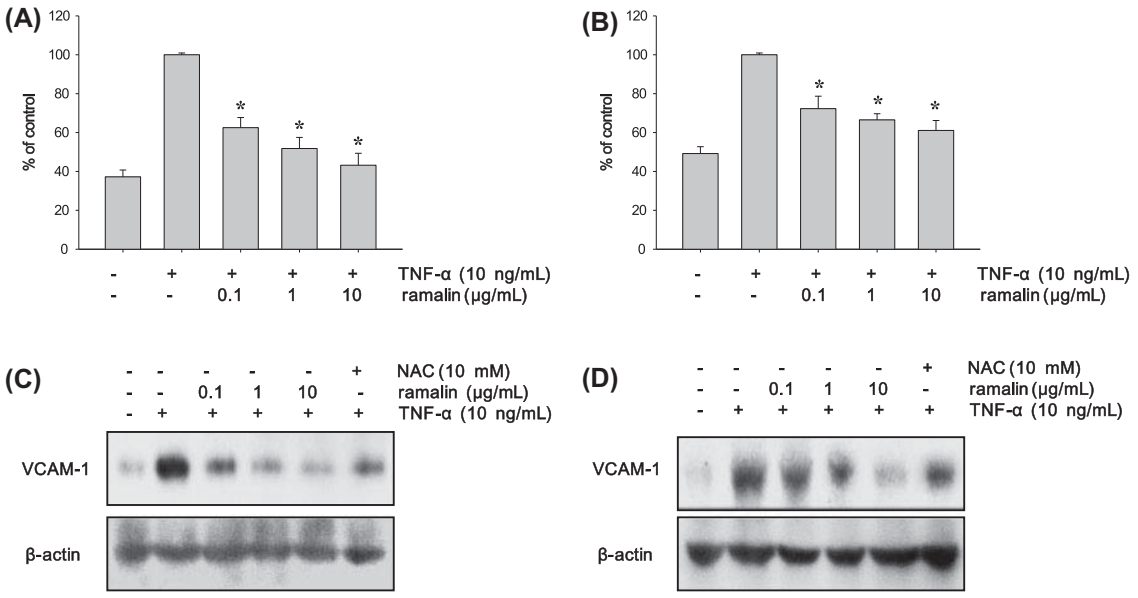

Fig. 8. Role of ROS in the inhibition of VCAM-1 expression by ramalin in TNF-α-stimulated VSMCs.

Notes: MOVAS-1 cells (A) and HASMCs (B) were pretreated with ramalin 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 ± SEM of quintuplicates from a representative experiment. *$p < 0.05$, significantly different from the group treated with TNF-α. MOVAS-1 cells (C) and HASMCs (D) were pretreated with NAC at the indicated concentrations for 2 h followed by the stimulation with TNF-α for 8 h. VCAM-1 expression was measured by western blot assay. The β-actin protein level was considered as an internal control. The results illustrated are from a single experiment, as a representative of three separate experiments.

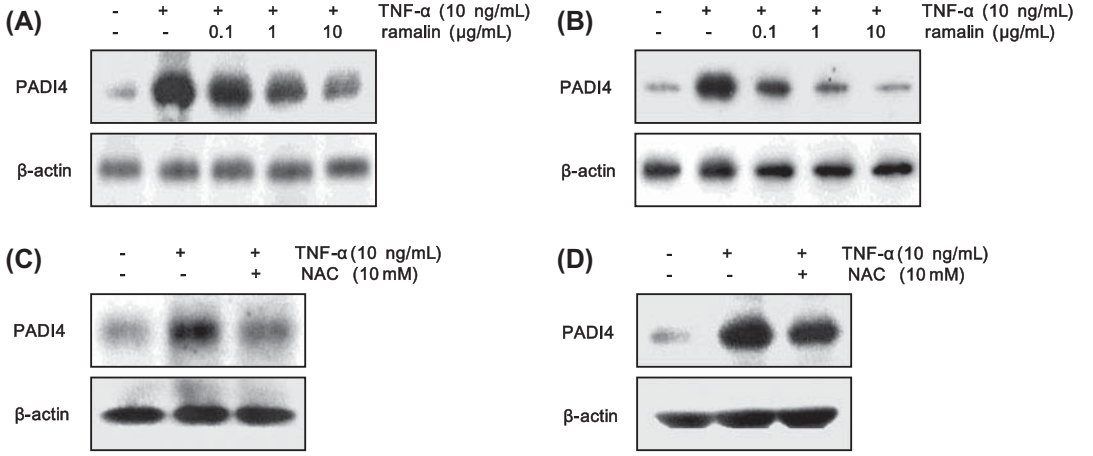

Fig. 9. Effect of ramalin and NAC on PADI-4 expression in TNF-α-stimulated VSMCs.

Notes: MOVAS-1 cells (A, C) and HASMCs (B, D) were treated with the indicated concentrations of ramalin or NAC for 2 h and then incubated with TNF-α (10 ng/mL) for 4 h. The whole cell lysates were analyzed by western blot. The β-actin protein level was considered as an internal control. A typical result from three independent experiments is shown.

enhance the transcription by activation of NF-κB and AP-1, leading to increased adhesion molecule expression.[49] This indicates that ROS can participate actively in cell signaling. We found that antioxidant NAC significantly reduced TNF-α-induced VCAM-1 expression. Based on these findings, the inhibitory effect of ramalin on the VCAM-1 expression and activation of NF-κB, AP-1, and MAPK induced by the treatment of TNF-α could be due to its antioxidant activity and the prevention of ROS production.

Recent studies have demonstrated that PADI4 plays an important role in gene regulation and inflammatory diseases.[28,29] In the present study, we identified a novel role for PADI4 involved in mediating the induction of VCAM-1 in VSMCs. TNF-α increased the expression of PADI4 via ROS induction and subsequent activation of NF-κB and AP-1, and up-regulation of VCAM-1 expression in VSMCs. However, ramalin inhibits PADI4 expression, thereby leading to inhibition of activation of NF-κB and AP-1, and up-regulation of VCAM-1 expression. These results suggest that ramalin treatment results in the inhibition of PADI4-mediated activation of VCAM-1.

In this study, it is worthwhile to note that pretreatment with ramalin significantly reduced the level of intracellular free Ca^{2+} increased by TNF-α in a concentration-dependent manner (data not shown). Previous studies have also shown that TNF-α increased intracellular Ca^{2+} concentration in lung micro vascular endothelium and Ca^{2+} mediated TNF-α-induced VCAM-1

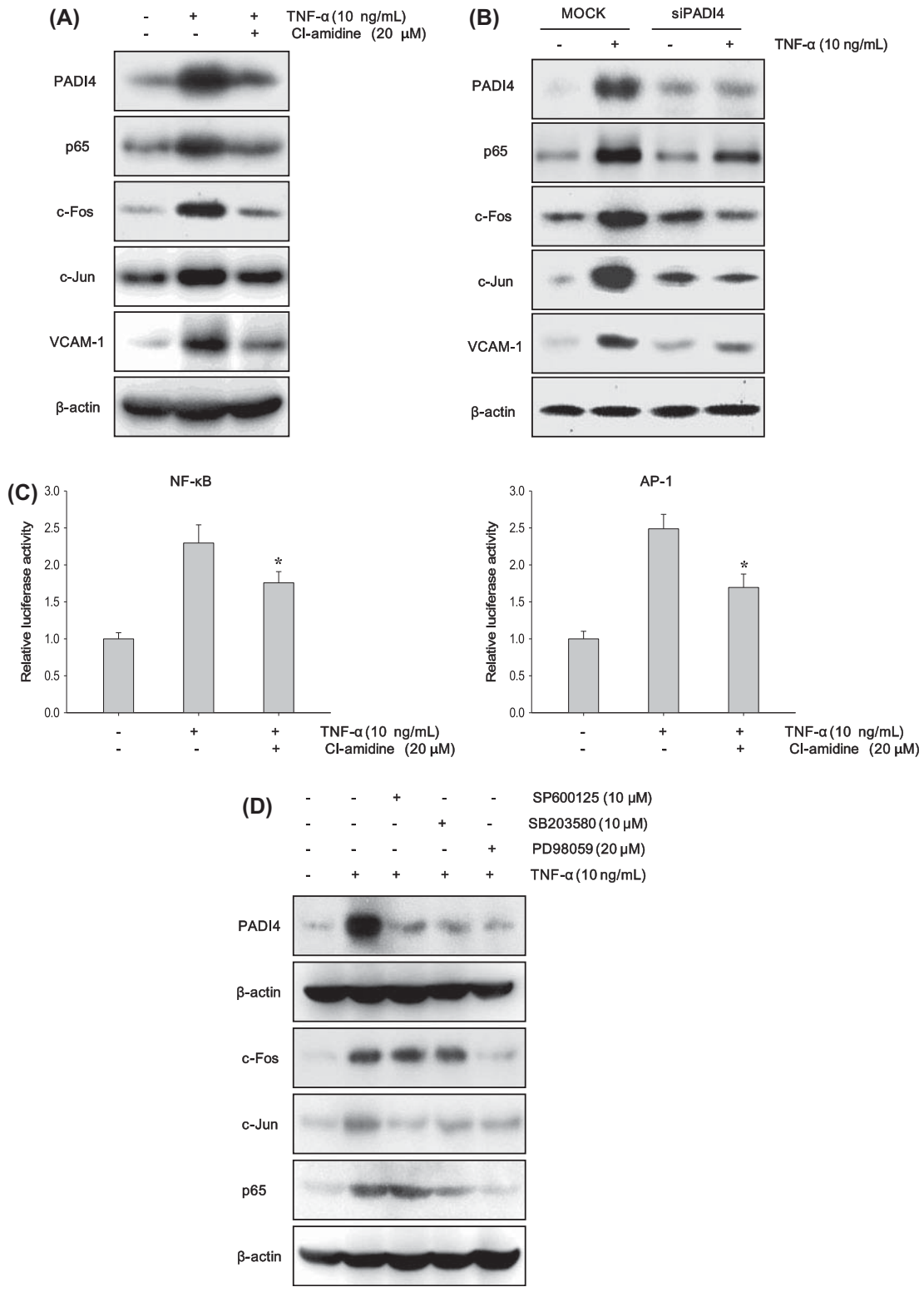

Fig. 10. PADI4 expression is required for AP-1 and NF-κB activation by TNF-α in VSMCs.

Notes: (A) MOVAS-1 cells were pretreated with the indicated concentration of cl-amidine, PADI4 inhibitor, for 2 h and then stimulated with TNF-α (10 ng/mL) for 4 h. (B) MOVAS-1 cells were transiently transfected with PADI4 targeting siRNA for 24 h followed by TNF-α (10 ng/mL) for 4 h. The whole cell lysates were analyzed by western blot. The β-actin protein level was considered as an internal control. A typical result from three independent experiments is shown. (C) MOVAS-1 cells were transfected with pGL3-NFκB-Luc reporter plasmid, pGL3–AP-1–Luc reporter plasmid, and pCMV-β-gal, pretreated with indicated concentration of cl-amidine (20 μM) for 2 h, and stimulated with TNF-α for 4 h. The results are mean ± SEM of 3 experiments. *$p < 0.05$, significantly different from the group treated with TNF-α-stimulated cells not treated with cl-amidine. (D) MOVAS-1 cells were pretreated with P38 MAPK inhibitor, SB203580 (10 μM), ERK1/2 inhibitor, PD98059 (20 μM), and JNK inhibitor, SP600125 (10 μM) for 2 h, respectively, and then stimulated with TNF-α (10 ng/mL) for 4 h. The whole cell lysates were analyzed by western blot. The β-actin protein level was considered as an internal control.

expression in tracheal smooth muscle cells.[50,51] These findings further support that PADI4, a calcium dependent enzyme, is involved in inhibitory effect of ramalin on VCAM-1 expression.

In summary, we demonstrate that ramalin is able to inhibit the expression of VCAM-1 in VSMCs through the suppression of MAPK and PADI4-dependent NF-κB and AP-1 signaling pathways via the induction of ROS. The cell surface expression of VCAM-1 suppressed by ramalin led to reduced adhesion of THP-1 cells to TNF-α-stimulated VSMCs. Therefore, the inhibitory activity of ramalin on VCAM-1 in VSMCs implies the possibility of a pharmacological intervention specifically directed toward inflammatory diseases including atherosclerosis.

Conflict of interest

The authors have declared that no conflict of interests exists.

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